The impacts of microplastic ingestion on marine polychaete worms

Submitted by Daniella Jane Hodgson to the University of Exeter as a thesis for the degree of Masters by Research in Biological Sciences

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For Nanny Rita
and Pete

The impacts of microplastic ingestion on marine polychaete worms.

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Abstract:

The benthic marine habitat is a sink for microplastics, however, our understanding of their impacts on marine organisms is still limited. This thesis investigates the ingestion and subsequent impacts of microplastics in the marine benthic dwelling polychaete worms, *Hediste diversicolor* and *Ophryotrocha labronica*. Firstly, microplastic ingestion by *H. diversicolor* in three estuaries across South Devon, UK, each of which were exposed to either high, medium or low levels of infrastructure and human population was assessed. The data showed 58.58% of *H. diversicolor* individuals ingested plastic-like particles, with fibres accounting for 86.8 % of all plastics observed. However, no significant differences in the amount of plastic-like particles ingested between sites were found. Microplastic fibres are the most commonly reported plastic shape in environmental samples, such as sediments, and during gut contents analysis of numerous phyla worldwide. However, the majority of research assessing the impacts of ingested plastics focus on microplastic spherical in shape. Therefore, the difference in toxicity between microplastic beads and fibres in *H. diversicolor* was investigated. The project found ingested fibres induced a greater oxidative stress response compared to that of microbeads and consequently caused cellular damage in the form of lipid peroxidation. Cellular repair and maintaining homeostasis is energetically expensive and in turn, may impact an individual's fitness. Therefore, the impacts of microplastic exposure on the feeding and fitness of *O. labronica* were assessed. *O. labronica* exposed to plastics produced less offspring and significantly smaller eggs than unexposed mating pairs, which ultimately could lead to deleterious impacts at the population level. However, the protein content of those eggs had a similar energetic content and consequently, there was no difference in the offspring survival rate.

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Definitions and abbreviations

Biofilm:	A layer of mucilage adhering to a solid surface containing a community of microorganisms.
Crystallinity:	The presence of three-dimensional order on the level of atomic dimensions.
Gametogenesis:	The production of gametes.
Macroplastics:	Plastic >5 mm
Microplastic:	Plastics <5 mm and >100 nm
Nanoplastics:	Plastic < 100 nm
Oogenesis:	The production of eggs.
Proteolysis:	The breakdown of proteins by the action of enzymes.

ASW:	Artificial seawater
DDE:	1,1-Dichloro-2,2-bis(p-chlorophenyl) ethylene
FITC:	Fluorescein isothiocyanate
HDPE:	High Density polyethylene
KOH:	Potassium hydroxide
LPS:	Lipopolysaccharide
MDA:	Maldionaldenhyde
NBT:	Nitrotetrazonlium Blue
NRR:	Neutral red retenion
PA:	Polyamide

PBDE: Polybrominated diphenyl ethers

PBS: Phosphate-buffered saline

PCB: Polychlorinated biphenyl

POPs: Persistent organic pollutants

PPT: Parts per thousands

ROS: Reactive Oxygen Speices

RPM: Rotations per minute

SFG: Scope for growth

SOD: Superoxide dismutase

TBARs: Thiobarbituric acid reactive substances

WWTW: Waste water treatment works

XO: Xanthine Oxidase

Chapter 1:

Literature review: Factors influencing the uptake and biological impacts of microplastics in marine biota.

1.1. Introduction

Marine plastic debris is of increasing environmental concern and although plastics bring considerable societal benefits, their inappropriate use and disposal have led to the contamination of marine habitats worldwide in which the opportunities for removal are restricted (Worm et al., 2017). Although the first modern plastic, Bakelite, was developed in 1907, mass production of plastics only started in the 1940s after the optimization of inexpensive manufacturing techniques (Cole et al., 2011). Since then plastic production has increased rapidly which currently exceeds over 335 million tonnes per annum (PlasticsEurope, 2017; fig. 1.1) and is expected to increase by an average of 9.2 million tons per year (Conkle et al., 2018). Plastic pollution is now a huge area of public concern, with a constant presence in media and with governments and major brands seriously investing in opportunities to reduce the quantity of plastic entering the oceans.

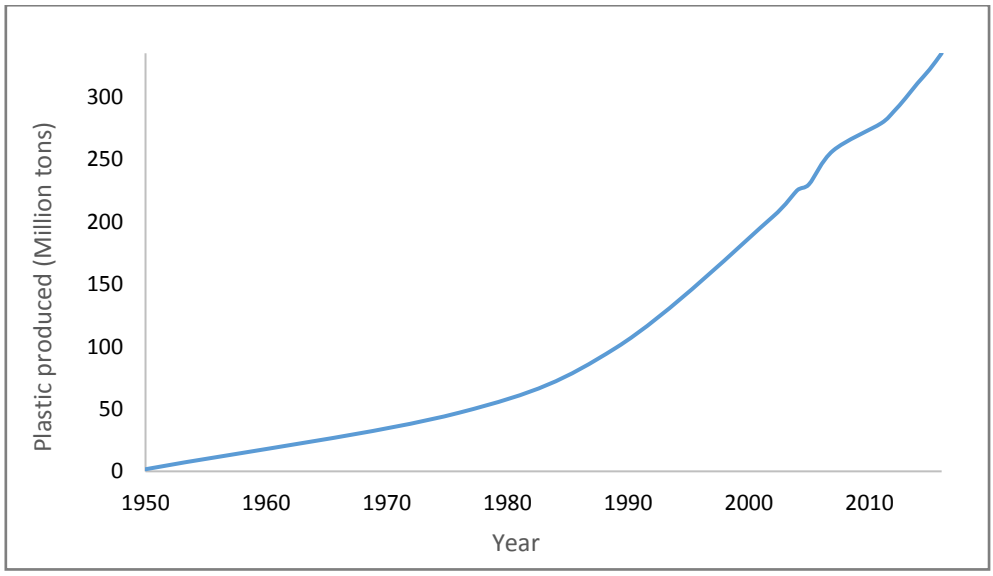

Figure 1.1: The increase of global plastic production in million metric tons/ year from 1950 to 2016. Data from *Plastic Europe – the facts, 2013-2016.*

Plastics are synthetic organic polymers mainly derived from oil or gas and typically incorporate a range of additive chemicals that increase functionality (Derraik, 2002; Thompson et al., 2009). They are strong, durable, lightweight and with such versatility and inexpensiveness, plastics are an ideal material to produce a large array of products. This mass production, along with poor waste management and durability, has led to the accumulation and persistence of plastics in marine habitats, generating considerable environmental challenges (Derraik, 2002; Cole et al., 2011) with 10% of annual production estimated to enter the marine environment (Avio et al., 2017).

Microplastic is a widely-used term that describes a heterogeneous assortment of particles, generally classified as <5 mm, that range in colour, shape and size (Thompson, 2015). The presence of these small plastic fragments has been documented in scientific literature since the 1970s (Carpenter & Smith, 1972) and since then their presence has been reported globally including deep-sea habitats (Bergmann et al., 2017; Courtene-Jones et al., 2017; Woodall et al., 2014) and Arctic sea ice (Obbard et al., 2014). The source of microplastics can be split into two categories; primary and secondary sources. Plastics manufactured to be of microscopic size for industry are defined as primary microplastics and are typically produced for countless uses in cosmetics and cleansers (Fendall & Sewell, 2009; Leslie, 2014), as air-blasting media (Gregory, 1996) and in medicine as vectors for drugs (Patel et al., 2009). Secondary microplastics derive from the breakdown of larger plastic items (macroplastics >5 mm) through the influence of UV radiation (Hidalgo-Ruz et al., 2012), mechanical (Cooper & Corcoran, 2010) and biological degradation (Hodgson et al., 2018) as well as a result of everyday use such as the release of fibres during the washing of synthetic clothing (Napper & Thompson, 2016).

Owing to their small size microplastics have a potential to be ingested by a wide range of marine organisms as they are within the optimal prey size range for many species across marine food webs (Galloway et al., 2017a). The ingestion of microplastics has been reported in 233 species (Law, 2017) and is evident across many different marine phyla that exhibit differing feeding strategies including deposit feeders such as polychaete worms (Wright et al., 2013a), suspension feeders such as barnacles (Goldstein & Goodwin, 2013) and mussels

(Setälä et al., 2016) which includes those intended for human consumption. For example, Van Cauwenberghe & Janssen (2014) reported the presence of microplastics in two commercially grown bivalve species. The common mussel, *Mytilus edulis,* had an average 0.36 ± 0.07 particles/ g $^{-1}$ and the Oyster, *Crassostrea gigas,* an average of 0.47 ± 0.16 particles/ g $^{-1}$ at the time of consumption. As a result, the authors concluded that European shellfish consumers could be taking up an average 11,000 microplastics/ year from shellfish alone.

There is an increasing amount of scientific literature on the effects of microplastic ingestion, most of which is carried out under controlled laboratory conditions and known quantities of plastics (Setälä et al., 2016; Auta et al., 2017). Effects include mechanical such as prolonged retention of microplastic (Welden & Cowie, 2016), reduced feeding rates (Watts et al., 2015) and digestive efficiency (McCauley & Bjorndal, 1999) and cellular effects such as inflammation (Wright et al., 2013a) and oxidative stress (Jeong et al., 2016). All of which could potentially impact energy uptake and allocation, and consequently lead to a reduction in growth and fitness. Microplastics also have the potential to act as a transport medium for harmful pollutants and additive chemicals (Hermabessiere et al., 2017). A recent review by Rochman et al., (2016) states that 245 studies have reported the biological impacts of marine debris, the majority of which focus on the organismal or individual level. These studies give an insight into the potential impacts of plastic ingestion, however, the extent of the biological effect of microplastic ingestion on natural populations is still limited (Thompson, 2015). This is likely due to the complex interactions between marine biota and their environment, and the fact most laboratory studies use plastic concentrations multiple times higher than those found in the marine environment.

The aim of this review is to discuss our current understanding of the impact of microplastic ingestion on marine biota, the potential factors that could affect their ingestion and to highlight the current gaps in research.

1.2. Abundance and distribution

Data on the quantification of plastic in the oceans is rapidly growing, however, the lack of standardization between studies creates difficulties when comparing data (Avio et al., 2017). Difficulties aside, the abundance and distribution of plastics has been reported in marine habitats on a global scale including estuaries (Sadri & Thompson, 2014; Naidoo et al., 2015), beaches (Mathalon & Hill, 2014; Ivar do Sul et al., 2017), the deep sea (Courtene-Jones et al., 2017) and in the open ocean (van Sebille et al., 2015).

A key aspect in regulating and reducing marine plastic debris is to understand its potential sources (Sadri & Thompson, 2014). Plastics can enter the marine environment through numerous routes originating from both marine and terrestrial sources (Auta et al., 2017). Land-based sources include lost material from landfills and dumps, accidental loss, general littering and during recreational use of the coast. This debris can then be transported into the oceans through multiple pathways such as in run-off, wastewater and by winds (Galgani et al., 2015). Wastewater treatment works are likely to be a major transport route of microplastics from land to sea (Murphy et al., 2016). For example, microplastics are used for many different functions in cosmetics and “down the drain’ products such as exfoliates in facewashes, tooth polishing in toothpaste, glitters in bubble bath and make-up, “optical-burring” effects in wrinkle creams, viscosity regulators and as bulking agents (Leslie, 2014). The US alone has been estimated to emit 263 tonnes of polyethylene microplastics every year, much of which are used in personal care products (Gouin et al., 2011). Likewise, the release of synthetic fibres from the washing of clothing into wastewater is another source of microplastics that have the potential to be transported into the marine environment. For example, Napper & Thompson (2016), found that an average 6 kg wash of acrylic clothing has the potential to release an estimated 700,000 fibres per wash. Due to their small size microplastics have the potential to bypass wastewater treatment process and enter aquatic environments. An assessment of the wastewater treatment works located on the Clyde Sea was calculated to release 65 million microplastics/ day into receiving waters despite previous treatment stages reducing the quantity of microplastics by 98.41% (Murphy et al., 2016). Efforts have been made to access the quantity of plastic entering the sea.

Jambeck et al., (2015), accessed the annual input of plastic into the ocean from waste generated by 192 coastal populations worldwide and estimated that out of the 275 million tons of plastic waste generated, 4.8 to 12.7 million tons of plastic entered the ocean in 2010. These estimates, however, are likely to under-represent the true amount of plastic entering the ocean as plastics from domestic wastewater was not taken into account in this study.

The majority of plastics entering the marine environment are likely from land-based sources (Galgani et al., 2011; Andrady, 2011; Sadri & Thompson, 2014), however, the research into ocean-based sources of plastic are more limited. Ocean-based sources of plastics include commercial and recreational fishing vessels, shipping, ferries and aquaculture (Galgani et al., 2015). Fishing gear is a commonly reported item of marine debris (see: Vieira et al., 2015; Pham et al., 2014; Fischer et al., 2015; Rummel et al., 2016) and although the release of plastic from vessels has been banned, losses are likely to still occur (Jambeck et al., 2015). It is estimated 6.4 million tons of material are lost from the fisheries industry into the oceans each year (Macfadyen et al., 2009), contributing to approximately 18% of marine plastic debris (Andrady, 2011). This fishing gear has the potential to break down and contribute to microplastic abundance in the marine environment.

Microplastics have been found from the equator to the poles (Browne et al., 2011) and from the shallows (van Sebille et al., 2015) to the depths (Van Cauwenberghe et al., 2013). Research initially focused on plastics found in oceanic gyres (Goldstein, 2012; Eriksen et al., 2013; Eriksen et al., 2014) but the researched areas have now spread and tend to include more coastal waters and fronts (Nor & Obbard, 2014; La Daana et al., 2016; van der Hal et al., 2017), and even more recently freshwater environments (Hendrickson et al., 2018; Imhof et al., 2018). This horizontal and vertical distribution of plastics demonstrates the magnitude of their contamination. For example, it has been estimated between 15 – 51 trillion particles (weighing 93,000 to 236,000 tonnes) of floating plastics are present across the world's surface oceans (van Sebille et al., 2015). These estimates however, are likely to be variable as the fate of a single particle of plastic can differ in copious ways. For example, 90% of these estimates are all based on sea surface measurements made using Manta trawls or Neuston nets with a minimum

mesh size of 333 µm. Therefore, the data is likely missing plastics smaller than 333 µm and the vast majority of plastics that have already sunk.

The buoyancy of plastic is likely to vary through its lifetime and ultimately affect its distribution and in turn its bioavailability. Sediments have now been identified as a major sink for marine microplastics (Goldberg, 1997; Woodall et al., 2014; Coppock et al., 2017). Once in the environment plastics can accumulate biofilms. This biofouling of plastic can affect its buoyancy and can cause positively buoyant plastics to sink (Kooi et al., 2017). Koelmans et al., (2017), predicted that 99.8% of plastic that has entered the oceans since 1950 has sunk below the ocean's surface layer. Another recent paper shows plastics have the potential to be incorporated into marine aggregates such as in faecal pellets and marine snows, which can act as a transport median of floating plastics to the sea floor (Porter et al., 2018).

In addition, abiotic factors such as currents, wind, geography and infrastructure affect the distribution of plastic, consequently making its distribution in the ocean heterogeneous (Salvador Cesa et al., 2017). This heterogeneity could create difficulties in accessing their fate and impacts in the marine environment. For example, Browne et al., (2010), measured the distribution patterns of macro - and microplastics along the Tamar River, Plymouth and found wind and density played an important role in the distribution of floating plastics with greater amounts of plastic, and denser microplastics found at sites downwind.

There have been legislations and actions taken by industry to reduce microplastics, for example with exfoliates in cosmetics and signal-use plastic carrier bags (Xanthos & Walker, 2017), Despite this, the amount microplastic is still likely to increase, even if plastic production was immediately ceased, due to the fragmentation of larger plastic items already present in the marine environment (Thompson, 2015). As its abundance increases so does its bioavailability to marine biota (Auta et al., 2017), and therefore potentially it impacts. The distribution and factors affecting its accumulation will also play an important role in which organisms will be affected (Lusher, 2015). Therefore, it is essential to develop an understanding of the fate and magnitude of the impact of microplastics in the marine environment.

1.3. Factors affecting uptake of microplastics

1.3.1. Size matters

Plastic debris comes in all shapes, types, and sizes and these factors must be considered when assessing their impacts on the marine environment. Owing to their small size, microplastic has an increased bioavailability to a larger range of organisms (Cole et al., 2011). These microplastics can be ingested by smaller organisms occupying low trophic levels and therefore have the potential to enter the food chain. A number of studies have now demonstrated microplastic ingestion by zooplankton species. For example, Cole et al., (2013) found 13 out of 15 zooplankton species tested readily consumed polystyrene beads 1.7 – 30.6 µm in size. In another study, Setälä et al., (2014) demonstrated the trophic transfer of microplastic spheres (10 µm) in planktonic organisms by offering a mesozooplankton community (prey) pre-exposed to fluorescent microplastic spheres to pelagic mysid shrimps, *Mysis relicta*. They found 100% of *M. relicta* individuals contained the fluorescent microspheres. Although the majority of research on microplastic ingestion is mainly undertaken on smaller organisms' evidence also suggests the ingestion of microplastic in larger biota. Besseling et al., (2015) found microplastics, consisting of 5 polymer types (polyethylene, polypropylene, polyvinyl chloride, polyethylene terephthalate and polyamide), in the intestines of a baleen whale (*Megaptera novaeangliae).*

The size of ingested plastic (macro to nano) could have substantial impact on its effect on an organism. Evidence from the field suggests the lethal effects of ingested macroplastics, such as data from stranded wildlife including whales (Unger et al., 2016), dolphins (Di Beneditto & Ramos, 2014), seals (Rebolledo et al., 2013) and turtles (Clukey et al., 2017). However, due to ethical issues the *in vitro* quantification of deleterious effects of macroplastic ingestion in marine organisms is fraught with issues. Therefore, to state plastic as the direct cause of death in these stranded animals is often an assumption.

Nanoplastics (<100 nm) are a newly emerging subject in the topic and although their study is the least researched area of marine litter it is suggested to be the most hazardous to marine life (Koelmans et al., 2015), with evidence reporting its

deleterious effects on marine biota (e.g. Bhattacharya et al., 2010; Wegner et al., 2012; Lee et al., 2013). Nanoplastics originate from both direct production and through the fragmentation of microplastics (Panel & Chain, 2016). Although microplastics (3.0 µm and 9.6 µm polystyrene particles) have been found to pass into the circulatory system of mussels (Browne et al., 2008). Bouwmeester et al., (2015), predicts that unlike microplastics, plastic nanoparticles may be more able to pass through cellular membranes and deeply penetrate organs, causing cellular damage. The translocation of plastics across the gut epithelium leading to systemic exposure is believed to be restricted to particles of <150 µm (Panel & Chain, 2016). For example, using hyperspectral imaging Galloway et al., (2017b) found nanopolymers (70 nm) delivered via food can be taken up across the gut epithelium and redistributed to the liver in larval zebrafish, however, the toxicological consequences of such translocation have not been established.

Numerous studies have been conducted to assess environmental concentrations and assemblages of plastic debris. For example, Claessens et al., (2011) found microplastics in all sediment samples analysed from harbours (166.7 ± 92.1 particles kg^{-1}dry sediment), beaches (92.8 ± 37.2 particles $^{kg-1}$ dry sediment) and sublittorally (97.2 ± 18.6 particles $^{kg-1}$ dry sediment) across the Belgium coast. Obbard et al., (2014), reported higher concentrations of microplastics in Arctic Sea Ice than many other highly concentrated areas, such as in the Pacific Gyre. However, inconstancies in data collection and difficulties in sampling technique and analysis may lead to under detention and misidentification of some plastic debris which is likely to increase with decreasing plastic particle size (Law, 2017). Lozano & Mouat, (2009), found trawls reported 100,000 times more microplastic using smaller 80 µm mesh compared to a 450 µm mesh. In order to conduct environmentally relevant exposure experiments, further research with more efficient sampling techniques are needed. Although the >100 nm/ <5 mm plastic debris size classes are generally used to define microplastics, there are no universally recognized definitions regarding plastic size (Van Cauwenberghe et al., 2015). These inconstancies in classification can be problematic when comparing data, therefore the creation of size standards for plastic is essential (Cole et al., 2011).

The investigation of microplastic ingestion and its effect is mainly limited to laboratory experiments (Wesch et al., 2016). Understanding the real-world effects of microplastic ingestion is fraught with multiple confounding factors that will also affect the health of an organism such as temperature, food, disease, salinity or other pollutants that would be unrealistically complex to disentangle. In addition to this, almost all data collected on the effects of microplastic ingestion has been from organisms exposed to unrealistically high concentrations of plastics, and therefore may not show real-world level effects. A recent study by Lenz et al., (2016) calculated that most experimental exposures are 2-7 orders of magnitude greater than the microplastic concentrations found in the marine environment. It is possible that environmentally relevant microplastic exposures could potentially lead to a reduced residence and retention time due to easier excretion of microplastic and could, therefore, reduce the potential of their effects. Research must now aim to gain measurements from environmentally relevant conditions to understand the extent of these effects.

In summary, there is some evidence, albeit mostly anecdotal, that plastic size may alter its impact. Larger plastic items have the potential to be lethal through entanglement (Gall & Thompson, 2015) and through physical damage via ingestion (e.g. Nelms et al., 2015). As macroplastic fragments into microplastics it appears to pass through an organism's digestive system more easily and in turn, the impacts become more sub-lethal. To date, there is no evidence of lethal impacts of microplastic ingestion at environmentally relevant concentrations but evidence that ingestion causes inflammation, oxidative stress and changes to feeding behaviour (For information on impacts see section 4). As the plastics fragment into even smaller nano-sized particles, they become small enough to pass across the gut lining and into tissues, where with continued exposure they can theoretically bioaccumulate increasing their potential to cause harm. It is therefore possible the effects of ingested plastic could be size dependent, with macro- and nano-sized plastics having a higher potential to cause deleterious effects. This evidence might be pulled together to formulate a size-dependent effects hypothesis for microplastics such as that presented in fig. 1.2. However, extensive investigation is needed to confirm this theory. Nonetheless, it poses interesting research questions for future study.

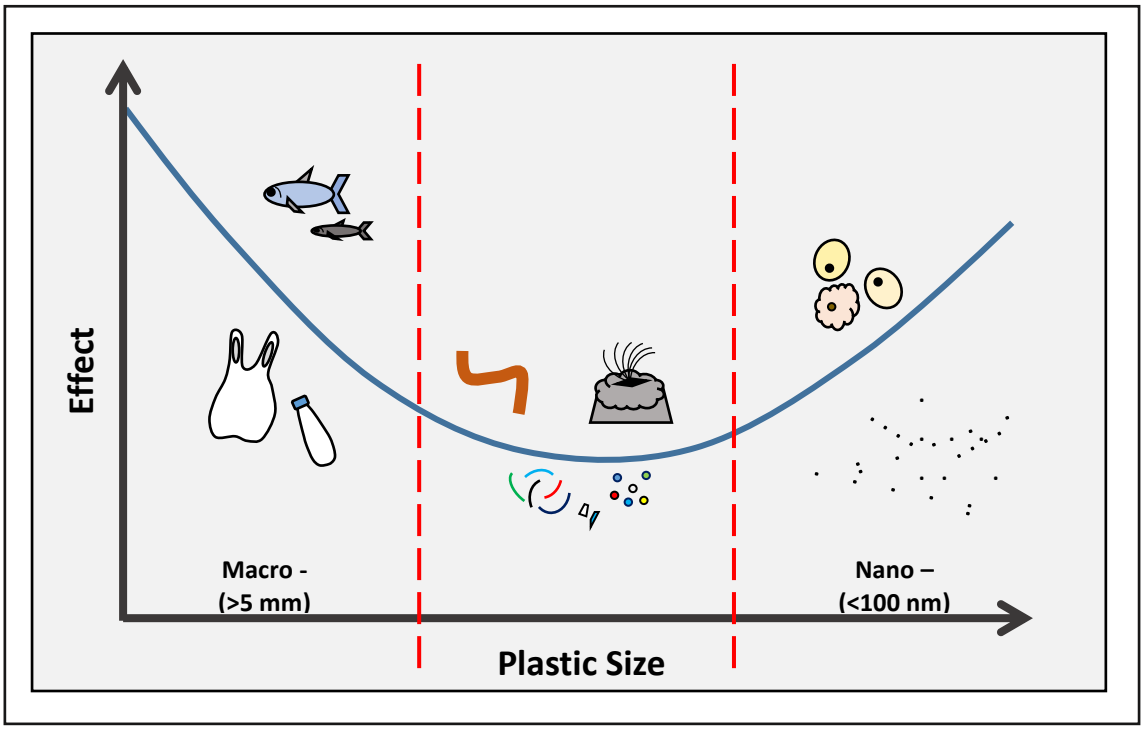

Figure 1.2: Diagram showing potential new paradigm for size-dependent effects of plastic debris on marine biota under environmentally accurate plastic concentrations from macro- to micro- and nano-sized plastics (*author's personal work*).

1.3.2. Shape

The shape of microplastic could also play an important role in the type and extent of impact on an organism (Wright et al., 2013b). Although the terminology may vary among researchers, microplastics are genuinely classified into 5 shape categories: fragments; fibres; beads; foams and pellets (Lusher et al., 2017). Fibers are the most commonly observed microplastic particles across almost all sampled habitats in the marine environment (see: Desforges et al., 2014; Gallagher et al., 2016; Naji et al., 2017), as well as in the intestinal tract of many marine species (see: De Witte et al., 2014; Nadal et al., 2016; Steer et al., 2017). For example, 68.3% of ingested plastic found within 10 species of teleost fish sampled from the English Channel were fibrous in form (Lusher et al., 2013), 87% of ingested plastic found within 3 deep-sea benthic invertebrate species were fibres (Courtene-Jones et al., 2017) and 96.5% of ingested microplastic in the brown shrimp, *Crangon crangon* analysed from North Sea coastal areas were

fibrous (Devriese et al., 2015). However, despite these findings, the majority of laboratory-based studies investigating the impacts of microplastic ingestion have used plastic spherical beads and granules (Phuong et al., 2016a) most likely due to these plastics being more convenient to obtain for studies.

The shape of a microplastic particle could influence how easily it is consumed and consequently how much plastic of a certain shape an individual may ingest. For example, an experimental exposure found the grass shrimp, *Palaemonetes Pugio,* to ingest significantly less microplastic fibres than beads (Gray & Weinstein, 2017). Another recent study investigated the dose-dependent effects of microplastic beads and fibres on the freshwater zooplankton, *Ceriodaphnia dubia*. Despite no evidence of microfiber ingestion, fibres were found to have the greatest deleterious effects including significantly reducing reproductive output. The authors report that during exposure *C. dubia* became entangled in the plastic fibres which in turn compromised their ability to swim (Ziajahromi et al., 2017). It is probable that plastic shape and size overlap in effect. For example, a large fibre is likely to be more problematic to consume than a small bead and vice versa.

Once ingested plastic shape could also influence the way in which it interacts with an organism's internal structures and other ingested plastics. For example, microplastic fibres have the potential to ball up into knots which in turn could create a blockage and/ or internal damage (Gray & Weinstein, 2017). The presence of balled-masses of fibres has been reported in wild-caught langoustine, *Nephrops norvegicus*, from the Clyde Sea Area, Scotland (Welden & Cowie, 2016). Microplastic fibres could also have a longer gut residence time than that of spherically shaped plastics and in turn, could potentially cause greater harm. A recent study examining the toxicological effects of microplastic particles and fibres on the freshwater amphipod, *Hyalellla Azteca,* found microplastic particles had no significant difference in egestion time compared to that of its normal food items. However, fibres showed a longer residence time and slower egestion rates. In addition to this, they report that microplastic fibres had a greater negative effect on growth and reproduction. The authors suggest the slower egestion rates could explain the differences in toxicity between the two shapes (Au et al., 2015). In contrast to this, Gray & Weinstein, (2017), found no

effect on plastic shape on gut residence time in the glass shrimp, *P. Pugio*. In summary, there remains a large knowledge gap on the role that size and shape play in the bioavailability and impact of microplastic ingestion which hampers our assessment of the risk that plastics pose to the marine environment. Future research should investigate the combined effects of microplastic size and shape to identify potential harmful combinations.

1.3.3. Polymer composition and length of time in the marine environment

Plastics comprise of a large assortment of materials which have a diverse composition and physicochemical properties, properties that will ultimately influence their fate in the marine environment (ter Halle et al., 2017). This will also affect how the plastic degrades and fragments, its buoyancy, which in turn may influence its distribution, its toxicity and potential to sorb and/ or release harmful chemical additives and pollutants (Andrady, 2017). Despite the different types of plastics that can be produced, approximately 90% of the global production is made up of only 5 types: polypropylene, polyethylene, polystyrene, polyethylene terephthalate and polyvinyl chloride (Andrady & Neal, 2009). These plastics, in varying quantities, are commonly found globally in the marine environment (See: Morét-Ferguson et al., 2010; Iñiguez et al., 2016; Wessel et al., 2016; La Daana et al., 2016; Lusher et al., 2015b; Imhof et al., 2017). Although most plastics themselves have low levels of toxicity there are some plastics (polystyrene, polyvinyl chloride, polyurethane and polycarbonate) that often contain hazardous additives and monomers that improve their functionality (Worm et al., 2017). The type of plastic ingested and associated additive chemicals could play a large role in the harm it poses to marine life.

Microplastics of differing material will have varying buoyancies in seawater. These differences in buoyancies will play a role in its distribution and in turn, influence what organisms the plastics will encounter (Andrady, 2017). On average seawater has a density of 1.02 – 1.029 g/ cm^3 (Enders et al., 2015). The majority of plastics produced have a density lower than that of seawater (Kukulka et al., 2012), such as polyethylene and polypropylene, and therefore tend to float, whereas denser plastics, such as polyvinyl chloride, will inevitably sink (Avio et al., 2017). However, there are many other factors that influence a microplastics

buoyancy such as biofouling load (Fazey & Ryan, 2016; Kooi et al., 2017), accumulation into marine aggregates (e.g. marine snow) (Long et al., 2015) and winds (Kukulka et al., 2012). For example, an assessment of microplastic ingestion by deep-sea organisms found evidence of ingested polypropylene fibres, a low-density plastic, in both a Sea Cucumber and Hermit crab. However, due to the low number of replicates analyzed during this study, the link between environmental abundances and ingestion of the low-density polymers could not be fully established (Taylor et al., 2016).

The chemical make-up and crystalline structure of plastics will play an important role in its weatherability (Andrady, 2017). As defined by Jones & Division, (2009), crystallinity is “the presence of three-dimensional order on the level of atomic dimensions”. Some plastics such as polypropylene, polyethylene and polyethylene terephthalate have a partially crystalline structure. Although crystallinity makes a material strong it can also cause it to become brittle. Over time the crystallinity and molecular structure of plastics can change and, in turn, affect the material’s density, strength, ease of oxidative degradation and fragmentation (and consequently size) and its potential to release harmful sorbed pollutants (Andrady, 2017). These changes will not only affect the distribution of the plastics but may also alter its toxicity compared to the original parent plastic (Lambert et al., 2017). It is therefore fundamental that future research considers the changing physiochemical composition of plastics over time when assessing the fate and subsequent toxicological effects of marine plastic debris.

In addition to this, there is increasing evidence that the length of time a plastic spends in the marine environment may also increase its palatability to marine organisms. Biofilms consist of a diverse community of microorganisms including bacteria, fungi, algae and protozoans. As soon as a plastic enters the environment it will be coated in inorganic and organic substances on which a biofilm layer can then form, something that can start to occur within minutes of entering the marine environment. Over time these biofilms will develop into complex communities (Rummel et al., 2017). It is possible the presence of biofilms may influence a plastic potential to be consumed. My previous work assessed the effect of biofilm load (clean or fouled) and plastic type (conventional/ degradable/ compostable) on the amount of plastic ingested by the

marine amphipod *Orchestia gammarellus.* We reported signs of plastic ingestion and shredding across all plastic types and fouling treatments with no effect of plastic type on the amount ingested over a 7-day exposure period. However, the presence of a biofilm significantly increased the amount of plastics consumed. During photographic analysis of the fouled samples, scaring marks, created by the amphipods mouthparts, were observed across the surface of the plastics suggesting these organisms are actively feeding on the plastic epi-biota. This data suggest that the presence of biological matter could act as a feeding cue to these organisms (Hodgson et al., 2018).

1.4. Impacts of microplastic ingestion

Plastic ingestion has now been reported in over 233 marine species (Law, 2017). However, our understanding of the effects of ingested plastics is still limited. The effects associated with microplastic ingestion are likely to be highly complex, impacting at differing levels of biological organization (Galloway et al., 2017a) and linked through numerous biological pathways that could lead to a cascade of effects that consequently impact an individuals' fitness (see: fig. 1.3), and in turn potentially population and ecosystems as a whole. There is a growing body of research demonstrating these effects with evidence suggesting that sub-lethal effects are more likely to occur than that of lethal ones (Worm et al., 2017).

1.4.1. Physical damage and blockages

Visible physical harm, such as lacerations, ulcerations and blockages, are often associated with larger macroplastic debris (Enders et al., 2015) ingested by organisms occupying high trophic levels such as marine mammals and birds. This kind of physical harm is likely to lead to highly deleterious impacts including death. However, determining death directly caused by plastic ingestion, especially in deceased subjects, is fraught with difficulties.

Although limited, there is evidence of ingested microplastics causing physical internal harm and blockages. For example, the sea bass, *Dicentrarchus labrax,* displays evidence of intestinal tissue alterations when fed on diets spiked with polyvinyl chloride pellets (<300 µm). After a 90-day exposure, *D. labrax* displayed

pronounced to severe tissue alterations, including changes in cell morphology and inflammation. In turn, this damage could compromise the intestinal function of this fish species (Pedà et al., 2016). Another more recent study found ingested microplastic of ~70 µm (polyamide, polyethylene, polypropylene and/ or polyvinyl chloride) caused intestinal damage in the zebrafish, *Danio rerio*, including cracking of the villi and splitting of enterocytes (Lei et al., 2018).

Due to their small size, it is probable that the potential for microplastics to cause blockages within the gut of an organism is less than that of larger plastics. However, once ingested microplastic fibres have an ability to "ball-up" and create a tangled aggregate of plastics which could have a greater potential to cause blockages and/ or be more problematic to egest. Such evidence has been found for this "balling" during experimental exposures in the shore crab *Carcinus maenas,* (Watts et al., 2015) and in wild-caught langoustine, *N. norvegicus* (Welden & Cowie, 2016). A field study by Murray & Cowie, (2011) reported that 83% of wild-caught *N. norvegicus* contained microplastics most of which was fibrous in form. They reported that over 50% of these specimens contained these tangled masses consisting of plastic fibres. Ultimately such blockages and increased retention times could lead to deleterious impacts on an organisms' health and fitness (Ziajahromi et al., 2017).

1.4.2. Cellular and sub-cellular effects

The threat of microplastic spans across all levels of biological organization from a sub-cellular and ecosystem level. Understanding these risks across all levels of organization is essential for the advancement of its management. Despite this, the majority of research has concentrated on effects at the individual level which often involves subcellular and cellular endpoints. Cellular level effects include cell damage, death and immune responses such as alterations in phagocytosis activity whereas subcellular effects include DNA damage, oxidative responses and enzyme activity (Galloway et al., 2017a). As mentioned in section *1.3.1*., there is increasing evidence that small plastic particles have the ability to move from the gut into tissues (Galloway et al., 2017b) and theoretically across cellular membranes and cause damage (Bouwmeester et al., 2015). For example, a recent study has shown polystyrene nanoplastics (mean diameter: 51 nm) can

penetrate the membrane surrounding the zebrafish, *D. rerio*, developing embryos and can accumulate within the embryos yolk sac. During development, these particles were found to have translocated and accumulated in the gastrointestinal tract, liver, heart, gallbladder, pancreas, and brain of these fish. No effect of exposure to these nanoplastics was found at a cellular level, however heart rate and swimming activity increased in exposed individuals (Pitt et al., 2018). In addition to this, there is increasing evidence of changes in an organism's immune response and cell health when exposed to microplastics. For example, after 96 hrs exposure to HDPE (<80 µm) the blue mussel, *M. edulis*, was shown to display a strong inflammatory response (indicative of an increased immune response) and lysosomal membrane destabilization (indicating poor cell health) (von Moos et al., 2012). Wright et al., (2013a), also found microplastic exposure (unplasticized polyvinyl chloride powder ~230 µm) increases the immune response in the lugworm, *Arenicola marina,* via increased phagocytic activity.

The ingestion of microplastics may also be harmful at the sub-cellar level affecting the way in which an organism can defend itself from oxidative stress. For example, Browne et al., (2013) found the lugworm, *A. marina*, exposed to polyvinyl chloride particles (230 µm) became >30% more vulnerable to oxidative stress than non-exposed individuals. In contrast, a study on 417 wild caught striped red mullet, *Mullus surmuletus,* found no evidence of cellular damage or oxidative stress in individuals that had ingested plastic (Alomar et al., 2017). These contrasting results could potentially be explained by the wild caught fish being exposed to lower levels of plastic and differences in the type of organism. Interestingly, another laboratory study assessing the harmful effects of microplastic ingestion in the marine copepod, *Paracylopina nana*, found size-dependent effects on Reactive Oxidative Species (ROS) production and consequently oxidative stress responses. They found individuals exposed to nano-sized plastics (0.05 µm) to have increased levels of ROS whereas individuals exposed to microplastic particles (0.5 - 6 µm) displayed ROS levels similar to that of non-exposure individuals. This reflected the activity of antioxidant enzymes produced by the copepods with the highest levels of activity found in individual's exposed to the nano-sized plastics (Jeong et al., 2017). This study helps to support our size-dependent effect theory of microplastics mention in section *1.3.1*.

1.4.3. Impacts on energy consumption

All life depends on the consumption of energy and metabolism of that energy (Amiard-Triquet et al., 2012). Plastic ingestion has the potential to impact an organism's energy consumption, evidence for which spans across different phyla including Arthropods (Cole et al., 2015; Watts et al., 2015), Chordata (Ryan, 1988) and Annelids (Wright et al., 2013a). Firstly, plastics present in the gut of an organism could lead to a false sense of fullness and ultimately reduce an organism's feeding rate. The ingested plastic could also cause nutrient dilution where non-nutritious items, such as plastic, take up space within the gut that could otherwise be filled with nutritious food (Ryan, 1988). In addition to this it is hypothetically possible that the presence of plastic within the food source, could also, by volume of food, dilute its nutritional content. Therefore, an organism with a plastic contaminated food source could be receiving fewer calories per volume of food than that of an organism consuming non-contaminated food. In addition to this, and although data is lacking, it is theoretically possible the presence of plastic could increase an organisms' feeding rate to compensate for a nutrient dilute diet. An increase in feeding could potentially lead to more energy reserves being spent on feeding and in turn giving less time available for other important functions such as reproduction (see: fig. 1.3). However, no study to date has reported such results.

A reduction in feeding rate, and/ or the dilution of nutritional food sources ultimately leads to an organism in up taking less energy. Consequently, a reduction in energy uptake could have deleterious impacts as less energy could be available for growth, repair and reproduction (see: fig. 1.3) (Cole et al., 2013; Wright et al., 2013a; Sussarellu et al., 2016). For example, Cole et al., (2015) found that the ingestion of 20 µm polystyrene beads significantly reduced the feeding rate and in turn the reproductive output of the pelagic copepod, *Calanus helgolandicus*. Plastics could also have prolonged retention time within an organisms' gut. A prolonged retention time, which potentially could alter with plastic shape and size, could potentially accelerate these effects (Au et al., 2015). In conclusion, it is possible a reduction in reproductive output, due to a reduction in energy uptake and/ or changes in energy budgets (see: fig. 1.3), could consequently lead to a reduction in a populations fitness (de Sá et al., 2015), and

ultimately impact ecosystems as a whole, which when considering the overall impact of plastic pollution is of significant concern (Galloway et al., 2017a).

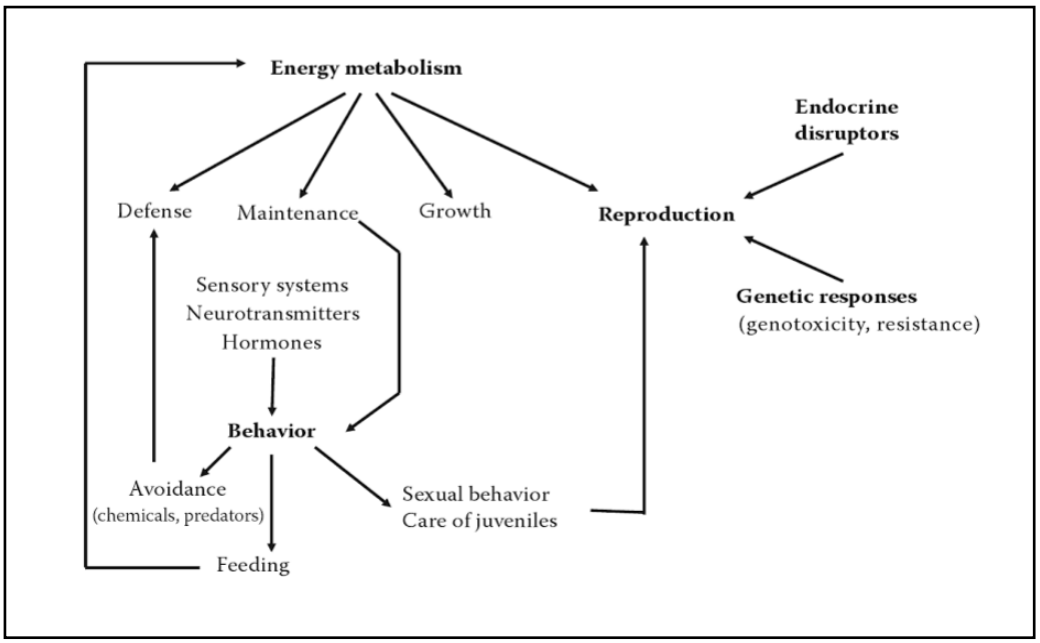

Figure 1.3: Flow diagram demonstrating the potential factors, and the linkages between them, effected by contaminates (Image taken from *Amiard-Triquet et al., 2012 – Ecological Biomarkers: Indicators of Ecotoxicology Effects.*

1.4.4. Chemical transfer

Harmful substances are added to some plastic to help increase performance. Such additives include brominated flame retardants (used to reduce flammability), phthalates (used as a plasticizer to soften plastics), nonylphenol (used as an antioxidant and plasticizer) and bisphenol A (a monomer used as an antioxidant) (Hermabessiere et al., 2017; Worm et al., 2017). These additives have the potential to be released from plastics once ingested and possibly accumulate in tissues (Hermabessiere et al., 2017). For example, a field study assessing polybrominated diphenyl ethers (PBDEs), an additive used in some plastics as a flame retardant, in short-tailed shearwaters, *Puffinus tenuirotris*, found a link between plastics containing these chemicals present in the bird's guts and the PBDEs analysed in the bird's abdominal fat. However, more observations are needed to confirm this link (Tanaka et al., 2013).

Plastic may also have the capability to absorb harmful chemicals, such as POPs and heavy metals, which are already present in surrounding seawater (Worm et al., 2017). Mato et al., (2001) used field-based experiments to assess whether polypropylene pellets absorb pollutants from the surrounding seawater. They found all pellets to have absorbed significant amounts of PCB, and DDE from the marine environment, which accumulated at concentrations up to 10^5 – 10^6 times greater than that of the surrounding seawater. The molecular make-up of polypropylene makes it nonpolar allowing for the absorption of hydrophobic pollutants through hydrophobic sorption. It is possible that plastic size could also play a role in the transfer of harmful pollutants to marine organisms. Smaller plastics have the potential to carry more harmful toxins than larger plastic by weight due to their large surface area to volume ratio (Song et al., 2015). Experimental exposures by Velzeboer et al., (2014) found the sorption of PCBs to nanoplastics was 1−2 orders of magnitude greater than to microplastics. However, in contrast, a recent review on the transport of chemicals via microplastic concluded that the overall input of these harmful chemicals from prey items could outweigh the influx from ingested microplastics and suggest that microplastics are unlikely to increase the exposure and therefore risk of these chemicals to marine organisms (Koelmans et al., 2016).

1.5. Conclusion

It is well known that microplastics are present globally across many marine habitats and their potential to be ingested by marine biota has been demonstrated across numerous phyla (Law, 2017; Worm et al., 2017). However, there are still many gaps in the research that need to be investigated to truly understand the impact of this debris in the marine environment. Firstly, it is important to understand the sources of microplastic, its concentrations and composition in the ocean, as well as the factors affecting its distribution. Such data can help identify the habitats most at risk and its availability to be ingested, which in turn can be used to create realistic experimental exposures. Secondly, it is essential to investigate how plastic size alters its impact. Understanding if the impact of microplastic ingestion is size-dependent will help to determine which organisms are most at risk and will aid in its management and to help build legislation.

Another large research gap in the subject is if the shape of microplastic impacts its effects. Fibres are, by far, the most commonly found plastic shape in the marine environment (see: Desforges et al., 2014; Gallagher et al., 2016; Naji et al., 2017), however, this is not reflected in the research investigating its effect. It is essential for future research to determine the difference in effect between shapes, and to use this to create experimental designs that are more environmentally realistic. In turn, this will create a larger picture of the overall impact of this debris. In addition to this, future research needs to focus on the potential for microplastics to be a vector of harmful pollutants and additives, which would be beneficial to investigate alongside the impacts of shape and size as these factors could potentially also impact the movement of these chemicals. Finally, research needs to start to consider the population and ecosystem level impacts of microplastic ingestion as the majority of work is currently focused at the cellular/ individual level. Therefore, future work should investigate how microplastic ingestion could affect energy intake and its consequential effects on reproduction. This would be especially interesting at a multi-generational level.

1.6. Aims of thesis

The aim of this thesis is to investigate the biological effects of microplastic ingestion in two polychaete worms. This project addresses the following novel hypotheses:

H1: Benthic polychaetes are ingesting microplastics in their natural habitats.

H2: Microplastic fibres exert a greater toxicity than microbeads via immune responses.

H3: Larger microplastic will exert a greater toxicity than smaller plastics via immune responses.

H4: Microplastic ingestion leads to fitness impacts via reduced reproductive output.

The harbour ragworm, *Hediste diversicolor,* will be used to assess the toxicity effects of microfibers compared to that of beads. *Ophryotrocha labronica*, will then be used to study the fitness impacts of plastic ingestion.

Chapter 2:

Assessment of microplastic ingestion by the harbour ragworm, *Hediste diversicolor*, across South Devon, UK.

2.1. Introduction

Recent evidence suggests that with time the majority of microplastic, including those positivity buoyant in seawater, will eventually sink and hence accumulate in the benthos (Kaiser et al., 2017; Katija et al., 2017; Porter et al., 2018; Ziajahromi et al., 2018). Marine benthic sediments are now considered a major sink for plastic debris (Goldberg, 1997; Woodall et al., 2014; Coppock et al., 2017), with plastic debris now reported in almost all benthic habits worldwide, such as beaches (Dekiff et al., 2014; Stolte et al., 2015), estuaries (Naidoo et al., 2015; Willis et al., 2017), mangroves (Barasarathi et al., 2011; Nor & Obbard, 2014), salt marches (Ball et al., 2016; Viehman et al., 2011) and the seafloor (Frias et al., 2016; Woodall et al., 2014; Bergmann et al., 2017), including the deep sea to depths of 10,898 m in the marina trench (Chiba et al., 2018). A recent assessment of deep-sea Arctic sediments discovered high levels of microplastic contamination (44 – 3,463 microplastic particles/ L^{-1}). They identified 18 types of plastic, the majority of which consisted of chlorinated polyethylene (38 %), polyamide (22 %) and polypropylene (16 %) (Bergmann et al., 2018), despite both polypropylene and polyethylene being positively buoyant in seawater (Avio et al., 2017). It is therefore likely that organisms forming part of the benthic community have a high potential to come into contact and interact with microplastic debris, and consequently could be at risk of harm. Thus, it is ecologically important to research the effects of microplastic on sediment-dwelling benthic organisms.

Despite the accumulation of plastics in the benthos, environmental data on microplastic ingestion tends to be heavily biased towards pelagic species such as zooplankton (Desforges et al., 2015), fish (Lusher et al., 2013; Nadal et al., 2016; Steer et al., 2017) and marine mammals (Besseling et al., 2015; Lusher et al., 2015a). For example, a recent study found 7 species of wild-caught

mesopelagic fish from the Northwest Atlantic to contain an average of 1.8 microplastic particles/ fish in their gut (Wieczorek et al., 2018). Evidence of microplastic ingestion in benthic species has been mainly collected from laboratory-based exposure studies, however, there are a few examples showing ingestion in wild benthic species. In one example, Davidson & Dudas (2016) found microplastic present within both wild and farmed Manila clams (*Venerupis philippinarum*) at concentrations ranging between 0.07 to 5.47 particles/ g.

The harbour ragworm, *Hediste diversicolor*, is an intertidal burrowing polychaete inhabiting brackish waters across Northwest Europe (Hayward & Ryland, 2017). They are an omnivorous species which display two feeding modes. The first as a deposit feeder, consuming both flora and fauna from surface sediments and secondly as a suspension feeder via a mucus web secreted by the worm. They are one of the most commonly found polychaete worms in British estuaries and are an important prey species for many marine and coastal organisms such as birds and fish (Fish & Fish, 2011). Microplastic ingestion by polychaete worms has previously been reported during laboratory-based exposure experiments with evidence of some deleterious effects. Wright et al., (2013a) found the lugworm, *A. marina*, to readily ingest unplasticized polyvinyl chloride (130 µm). After a 10-day exposure to these microplastics, *A. marina* displayed reduced feeding activity and an increase in immune function measured as phagocytic activity. However, data on microplastic ingestion by *H. diversicolor* is very limited, with only one other published study (Gomiero, et al., 2018), and despite being an ecologically importance species, the ingestion of microplastic has yet to be determined in wild populations.

The aims of this chapter are to determine microplastic ingestion in wild populations of *H. diversicolor* at multiple estuaries across South Devon, UK, which exhibit low, medium and high levels of potential plastic contamination. In addition, the shape, colour and size composition of these ingested plastics will also be investigated.

2.2. Methods

2.2.1. Sample sites and Hediste diversicolor collection

Three estuarine sites with established *Hediste diversicolor* populations were chosen across South Devon, UK, for both their potential level of contamination and ease of access. The sites were categorized as representing high, medium or low probability levels of contamination which was assessed by number of wastewater treatment works (WWTW) that discharge into the estuary, the human population of their associated catchment areas (see: Table 2.1), surrounding industrial infrastructure and human settlement (see: fig. 2.1), as well as litter observations. The Plym Estuary, Plymouth (50°22’22.3” N 4°06’10.6” W) was classed as the high level site, the Exe Estuary, Exton (50°40’03.1” N 3°26’39.2” W) was classed as the medium site and Kingsbridge Estuary, Bowcombe Creek, Salcombe (50°16’36.9” N 3°45’37.8” W) was classed as the site with the lowest levels of potential contamination.

Using a garden fork, 30 *H. diversicolor* specimens were collected from the mid-shore at each site during low tide in July/ August 2017. Each worm was thoroughly washed with filtered 22 ppt ASW and transferred into individual 50 ml falcon tubes. Upon arrival at the laboratory the worms were weighed, then snap frozen in liquid nitrogen and stored at -20 ° C until analysis.

Table 2.1: Waste Water Treatment Works (WWTW) discharging into sample sites with the associated catchment area population (WWTW discharging into sea are disregarded). (South West Water, 2018 *in conversation*).

Sample site	WWTW	Population of catchment area
Plym Estuary	Marsh Mills	59,245
	Central	107,931
		Total: ***167,176***
Exe Estuary	Countess Wear	137,000
	Lympston	1,496
		Total: ***138,496***
Kingsbridge Estuary	Kingsbridge	6,669
	East Portlemouth	31
	West Charleton	441
	Malborough	2,578
		Total: ***9719***

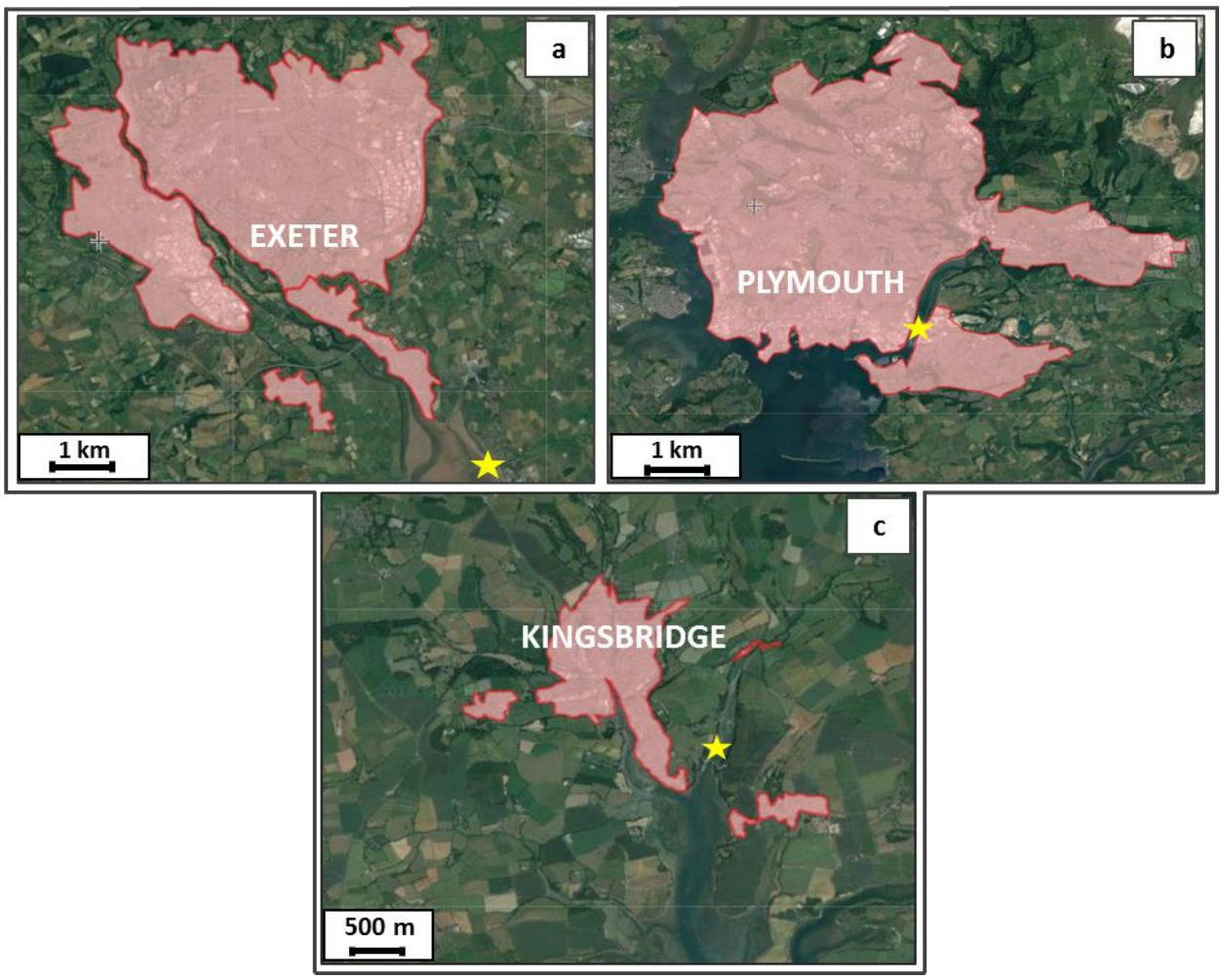

Figure 2.1: Map showing infrastructure and human settlement (*areas in red*) in surrounding areas of sample sites (*yellow stars*). *(a) Exe Estuary (b) Plym Estuary (c) Kingsbridge Estuary.*

2.2.2. Tissue digestion and microplastic analysis

Twenty-five millilitres of 10% filtered potassium hydroxide (KOH) was added to each falcon tube containing a *H. diversicolor* individual and was left to digest at 60 °C. After 12 hrs the digested worms were vacuum filtered through 10 µm Cyclopore® polycarbonate membrane filters. Filter papers were then transferred into petri dishes and sealed with parafilm to prevent any contamination. All equipment was thoroughly washed with ultra-pure water and analysed under a laminar flow fume hood to reduce contamination. Procedural controls consisting of blank filter papers were placed in the fume hood to control for contamination from atmospheric plastic during digestions and filtering. Prior to use all glassware was acid washed and all tools cleaned using filtered ultrapure water and ethanol

to prevent cross-contamination of plastics. The filtered sample from each worm was then visually examined under the Olympus microscope for particles synthetic in appearance by scanning the filter papers at 3.2 and then 1.6 magnification for 5 minutes each. Identification techniques described by Lusher et al., (2017) were implemented where synthetic particles were examined for the absence of a cellular structure and homogeneous in colour, gloss and thickness. The analysed plastic-like particles where then categorized by shape as either a fibre, granule, bead, fragment, foam or sheet and then the plastics length and width were measured using ImageJ.

2.2.3. *Statistical analysis*

All data were analysed using the statistical package MINITAB 16. Data were first tested for normality using the Kolmogorov-Smirnov test. If normal, the data were analysed using a one-way ANOVA. However, if non-normally distributed, data were log transformed and retested for normality. In the case of re-occurring non-normality the non-parametric test, Kruskal-Wallis, was utilized.

2.3. Results

In order to assess whether there is an influence of worm size on the number of plastic-like particles ingested by *H. diversicolor* a linear regression of all wet worm weights (g) from all sites was conducted. This found no relationship between worm weight and plastic-like particles ingested by individuals (R^2 = 0.21; F_{88} = 0.18; p = 0.671; fig. 2.2). Therefore, all data is presented here as plastic-like particles per individual.

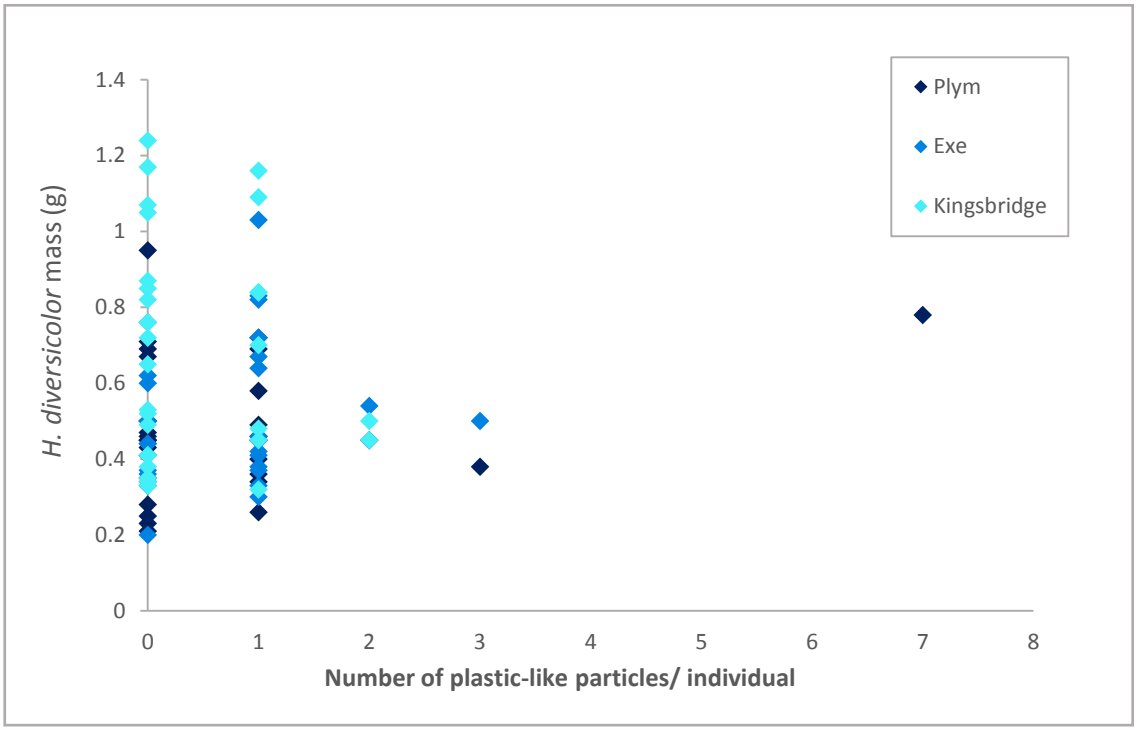

Figure 2.2: Number of plastic-like particles per individual versus *H. diversicolor* mass (g) showing no relationship.

A total of 53 (58.89 %) of the *H. diversicolor* sampled where observed to contain plastic–like particles, with 12 individuals (40%) of worms at Kingsbridge Estuary, 19 individuals (63.33 %) at Exe Estuary, and 22 individuals (73.33 %) at the Plym Estuary retrospectively. There was no significant difference in the number of plastic-like particles per individual *H. diversicolor* between sites (*Kruskal-Wallis,* H (2) = 2.03, *p* = 0.362; fig. 2.3*)*, with the average number of plastic-like particles ingested (from lowest to highest) being 0.4 ± 0.11 particles/ individual (Kingsbridge), 0.63 ± 0.13 particles/ individual (Exe) and 0.733 ± 0.25 particles/ individual (Plym).

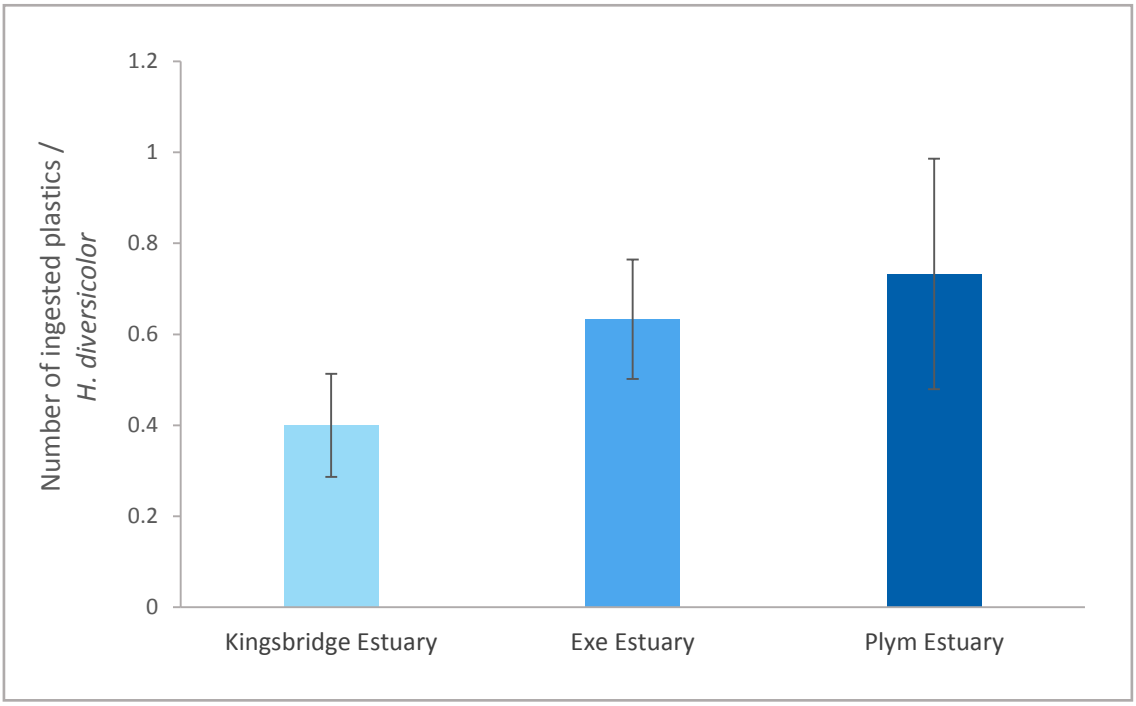

Figure 2.3: The average number of plastic-like particles ingested/ *H. diversicolor* individual between each sample site.

Fibres made-up the majority of plastic-like particles found, accounting for a total 86.8% across all sites (Kingsbridge (75 %), Exe (89.47 %), Plym (90.99 %) (fig. 2.4; Table: 2.1). The average length of the fibres from each site were; Kingsbridge: 1553.93 µm ± 428.9, Plym: 1173.12 µm ± 211.46 and Exe: 1246.72 µm ± 201.31 (fig. 2.5; Table 2.2).

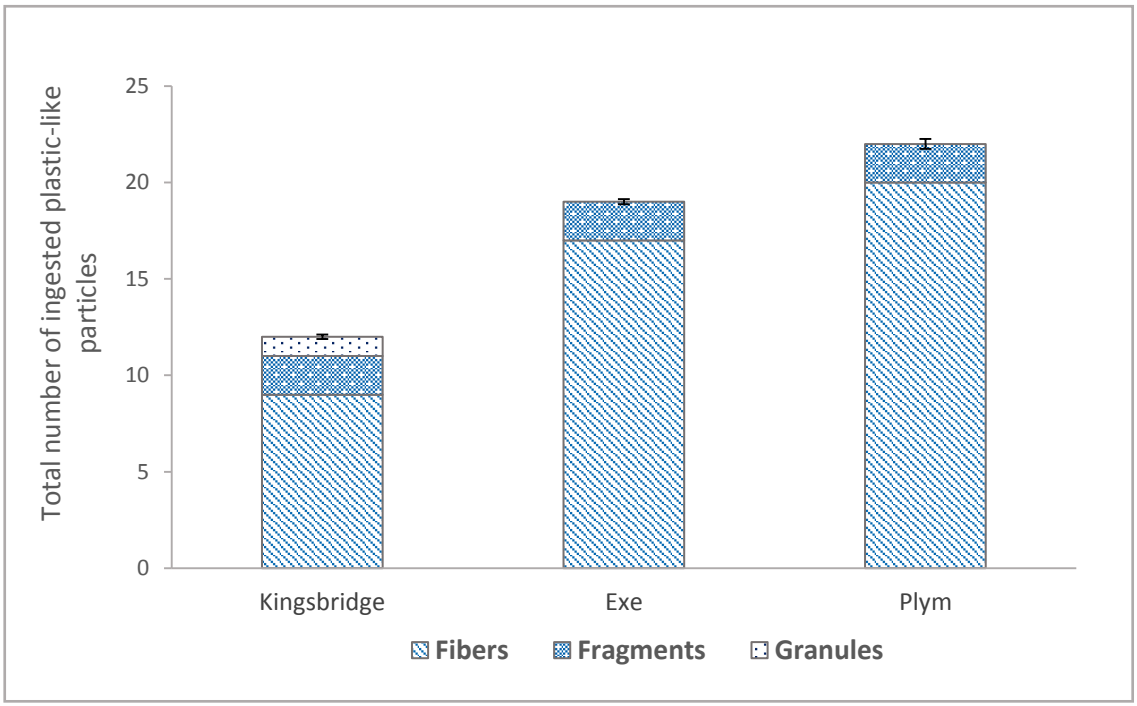

Figure 2.4: The total amount of ingested plastic-like particles by *H. diversicolor* between each sample sites with details showing the composition of microplastics shapes (fibres; fragments; granules).

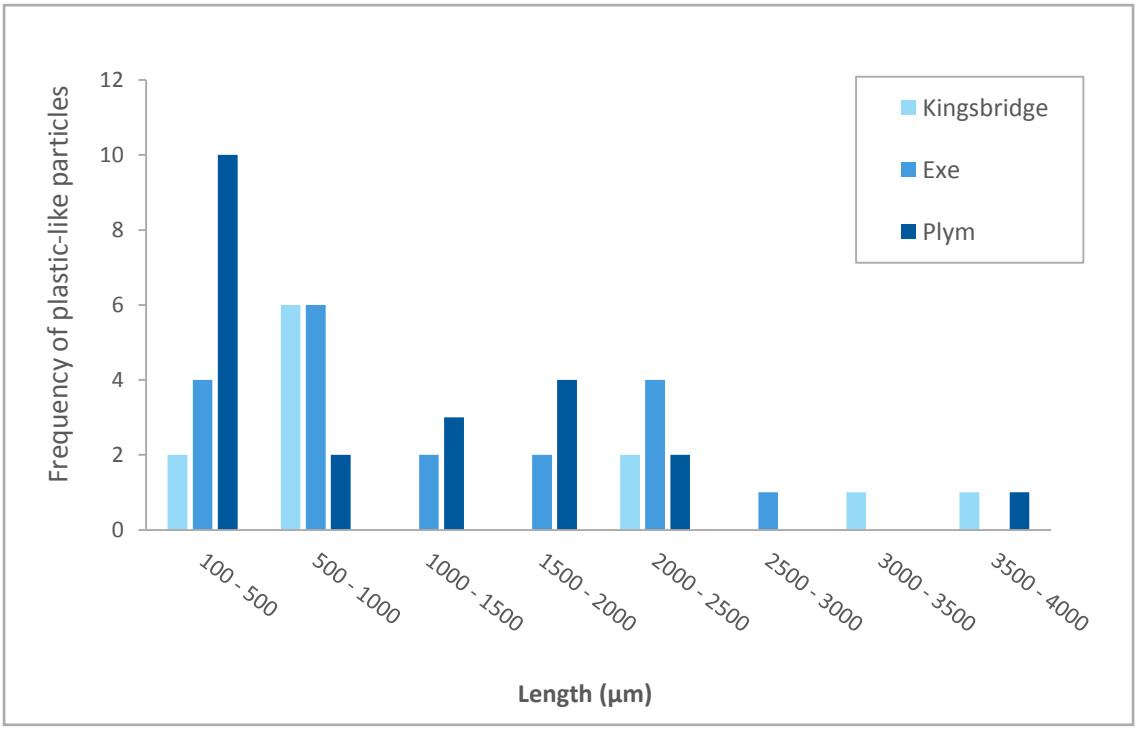

Figure 2.5: Size frequency of ingested plastics-like particles by *H. diversicolor* across each sample.

***Table 2.2*:** Descriptive data on ingested plastic-like particles colour, shape and size between sites.

	Kingsbridge Estuary	*Exe Estuary*	*Plym Estuary*
Colour			
Black	1 *(8.33 %)*	3 *(15.79 %)*	4 *(18.18 %)*
Blue	8 *(66.66 %)*	4 *(21.05 %)*	7 *(31.82 %)*
Clear	1 *(8.33 %)*	9 *(47.37 %)*	4 *(18.18 %)*
Red	2 *(16.66 %)*	3 *(15.79 %)*	7 *(31.82%)*
Shape			
Fibre	9 *(75 %)*	17 *(89.47 %)*	20 *(90.99%)*
Fragment	2 *(16.66 %)*	2 *(10.53 %)*	2 (*9.09%)*
Granule	1 *(8.33 %)*		
Length (*µm*)			
Fibre	1553.93 (± 428.91)	1246.72 (± 201.31)	1173.12 (± 211.45)
Fragment	653.68 (± 114.32)	132.833 (± 27.43)	239.60 (± 14.05)
Granule	239.99± 0)		

2.4. Discussion

This study clearly demonstrates microplastic ingestion in three wild populations of *Hediste diversicolor*. In total, over half the individual worms analysed (58.89 %) contained plastic-like particles. No relationship between *H. diversicolor* wet weight (g) and the number of plastic-like particles ingested was found, hence there was no rationale for normalising this data by worm weight. Respiration and feeding rate are more than often standardized to body weight as it is well established that these parameters change proportionally with body size in an allometric relationship. However, research has not yet established whether body size affects microplastic uptake. Despite this, some researchers studying the ingestion of plastics normalized their data by weight under the assumption that this holds true (For example see: (Hämer et al., 2014; Van Cauwenberghe &

Janssen, 2014; Van Cauwenberghe et al., 2015; Courtene-Jones et al., 2017; Waite et al., 2018). It is possible that microplastic encounter rate could potentially be a more important influence on uptake than that of organism size. For example, habitat type could be an influential factor in how much microplastic an organism could encounter. In addition to this, feeding mode could also play an important role in microplastic consumption. Further investigation is needed to assess the relationship between body size and microplastic uptake for other species.

This study found no difference in the observed number of ingested plastic-like particles per *H. diversicolor* individual between the sample sites. However, the data shows a trend towards higher levels of ingested plastic-like particles from individuals inhabiting sites with higher levels of potential contamination with *H. diversicolor* from the Plym observed with the greatest number of plastic-like particles (fig. 2.3). The number of plastic-like particles per gram of *H. diversicolor* observed in this current study (0.72 ± 0.23 to 1.48 ± 0.42 particles/ g) is found to fall in-line with data from Van Cauwenberghe et al., (2015) which assessed the microplastic ingestion in two wild-caught benthic species across six locations along the French - Belgian – Dutch coast. They found blue mussels, *M. edulis*, to have ingested an average of 0.2 ± 0.3 microplastics/ g and *A. marina*, 1.2 ± 2.8 particles/ g. Another study, however, found the wild freshwater annelid worm, *Tubifex tubifex*, to contain 29 ± 65.4 particles/ g tissue, which is a concentration 40 to 70 times higher than what was found in the current study (Hurley et al., 2017).

It would be expected that microplastics found in rivers and estuaries would be found in greater quantities in areas of high population and anthropogenic activity, For example (Gallagher et al., 2016) found the number of microplastic analysed in the Solent estuarine complex, UK, closely mirrored that of industrial inputs, and at points where rivers meet. In turn, larger quantiles of plastics in areas of input may increase the potential for organisms to encounter and ingest greater amounts of plastic. For example, a recent study assessing plastic ingestion by the longear (*Lepomis megalotis*) and bluegill (*Lepomis macrochirus*) sunfish in the Brazos River Basin, Central Texas, USA, found highest concentrations of ingested plastics from fish caught in urban sites, with downstream sites reporting the second highest level and upstream site with the least (Peters & Bratton,

2016). The accumulation of microplastics could be affected by many factors such as currents, tides, winds (Salvador Cesa et al., 2017). It is, therefore, possible that such factors could have outweighed the influx of microplastic in the current studies sample sites and affected its accumulation. For example, (Alomar et al., 2016) assessed the differences in microplastic accumulation in sediments at sites across areas of differing anthropogenic activity and reported the highest level of microplastics (0.90 ± 0.10 particles/ g) in a marine protected area, the site of least anthropogenic activity in the study. This suggests the transportation of microplastics from their source input to other areas in the marine environment. Understanding the sources, movements and settlement of microplastics will in turn aid research in assessing the impact of plastic debris in the marine environment.

Fibres were the most commonly observed ingested particle shape across all sample sites accounting for 86.8% of plastic-like particles analysed. This data falls within the same percentage range as other studies assessing the composition of microplastic in benthic dwelling organisms. For example, Courtene-Jones et al., (2017) reported an average of 80% of ingested plastics where fibrous in form in three species of benthic macroinvertebrates each of which exhibited different feeding modes. Despite these findings, very little research has assessed the impact of ingested microfibers on benthic dwelling organisms. It is essential future research assesses the impacts of ingested microplastic fibres rather than beads as their occurrence in the marine environment evidently outweighs that of any other shape. Unfortunately due to time restraints and access to equipment, FTIR analysis of the plastic-like particles was not conducted. Whist FTIR would have confirm the proportion of these particles were plastics as opposed to anthropogenic cellulose such as cotton, which have been reported to account to up to 50% of fibres, one of this studies main aims was to assess shape of ingested particle rather than polymer composition.

Wastewater treatment plants can act as an entry point for microplastic into aquatic environments (Talvitie et al., 2017) and are increasing assessed for their efficiency in microplastic removal (for examples see: Talvitie et al., 2015; Carr et al., 2016; Murphy et al., 2016; Mintenig et al., 2017). For example, one study

found a wastewater plant in Långeviksverket, Sweden to release 1770 microplastic particles/ hour, all of which were fibrous in form, despite more than 99% of particles being retained (Magnusson & Norén, 2014). The release of synthetic fibres from textiles is considered to be a major source of microfibers. These fibres can be released during the washing of clothing and consequently enter aquatic environments via wastewaters (Boucher & Friot, 2017). For example, De Falco et al., (2018) estimated a 5kg wash of polyester fabric could release over 6,000,000 fibres. As the plastic-like particle polymer type were unable to be assessed in the current study, it is unreasonable to infer the source of these particles. However, other studies often find ingested fibres such as nylon, polyester and acrylic (Li et al., 2016; Lusher et al., 2015a; Neves et al., 2015), which are materials commonly used in synthetic textiles (Salvador Cesa et al., 2017).

There is an increasing number of methods for detecting microplastic in environmental sediment samples including density flotation, stains, elutriation and sieving all of which have different recovery rates (see review: Miller et al., 2017). A recent method developed by Coppock et al., (2017) uses a density separation technique with an efficiency of 95.8%. The method uses a zinc chloride solution at a density of 1.5g/ cm^3 in a sediment-microplastic isolation unit to float plastics out of sediments. Due to time constraints during the current study, the sediments from the three sample sites could not be assessed for microplastic content using this technique. It would be advantageous for future studies to assess the microplastic content of sediments in order to provide comparisons between sediment plastic load and ingested plastic by sediment-dwelling organisms.

2.5. Conclusion

This study clearly demonstrates the polychaete harbour worm *H. diversicolor* readily ingested plastics in the wild, most of which are fibrous in form. However, no effect of wastewater treatment works input and catchment area population size on number of plastic-like particles ingested by *H. diversicolor* was evident. This data reinforces the ecological importance of researching the impacts of microplastic ingestion in these animals, especially that of plastics fibrous in form.

Chapter 3:

Ingestion of microplastic fibres induces a mild oxidative stress response in the benthic Polychaete, *Hediste diversicolor.*

3.1. Introduction

Understanding the biological impact of microplastic ingestion is a pressing issue, given its prevalence in the ocean. However, the level of impact that microplastic ingestion has on marine biota in real-world scenarios remains unclear. Evident from numerous assessments of plastic debris contamination in the marine environment, the composition of microplastic is heterogeneous consisting of particles differing in size, shape, colour and molecular composition. Such properties have the potential to alter the way in which micoplastics interact with the surrounding environment, affecting its bio-availability and ultimately its impacts on marine biota (Galloway et al., 2017a). However, although microplastics are comprised of this heterogenic soup of particles the majority of research is limited to the laboratory (Wesch et al., 2016) and assesses single polymer types, size and shapes, the majority of which are beads (Phuong et al., 2016b), often at concentrations magnitudes greater than that found in the marine environment (Lenz et al., 2016).

In the work of Chapter 2, I demonstrated that microplastic fibres are the most commonly found ingested plastic shape in the ragworm *Hediste diversicolor*. Such a finding is also often reported by other studies. For example, Nadal et al., (2016) found microplastic fibres to make up 100% of plastics analysed in the gastrointestinal tracts of the bream species, *Boops boops*, sampled around the Balearic Islands in the Mediterranean Sea. In another study, Li et al., (2016) reported fibres contributed to 65% of plastic ingested by the mussel, *Mytilus edulis*, sampled across 22 sites along the China coastline. However, this occurrence of microplastic fibres is not reflected proportionally in research assessing the ingestion of these debris. Fibres may have the potential to interact with an organism differently compared to spherically shaped plastics once ingested and in turn exert a difference in toxicity. For example, inhaled fibre-like

particles such as asbestos, are well known to cause disease in humans (Donaldson & Tran, 2004; Boulanger et al., 2014; Norbet et al., 2015) often as a result of persistent inflammation and oxidative stress caused by the fibres (Alfonso et al., 2015). There is an increasing amount of research showing induced immune and oxidative stress responses due to ingested microplastic beads. One example showed juvenile European sea bass, *Dicentrarchus labrax*, exposure to microplastic beads (1-5 µm) had an increase in lipid oxidative damage in both brain and muscle tissue, which is indicative of oxidative stress (Barboza et al., 2018). Another study reported inflammation and increased activity levels of the antioxidants superoxide dismutase and catalase in Zebrafish, *D. rerio*, exposed to 5 µm plastic beads (Lu et al., 2016). However, to the best of our knowledge, no study has yet compared the differences in these sub-cellular and cellular level effects between microplastic beads and fibres.

The benthic polychaete worm *H. diversicolor* readily ingests microplastics in the field, the majority of which were found to be fibrous in form (as reported across three populations in chapter 2). Therefore, the aim of this chapter is to assess whether microplastic fibres exert a different toxicity than microplastic beads when ingested by *H. diversicolor* by assessing its immune and oxidative stress responses when exposed to these microplastics.

3.2. Materials and Methods

3.2.1. *Sediment and worm collection*

The polychaete worm, *Hediste diversicolor* and sediment for the experimental exposure were collected from the mid-shore at low tide from the Exe Estuary, Exton, Devon (50°40'05.2"N 3°26'39.1"W). Only sexually immature specimens larger than ~4 cm were selected. Once collected, individuals were washed to remove excess sediment and placed in holding tanks containing 22 ppt artificial seawater (ASW; Tropic Marine salts) in temperature controlled rooms at 15 °C and fed trout pellets every 3 days. Sediment for the experimental exposure was sieved to 1 mm to remove gravel and unwanted biota, rinsed and left for 12 hrs to settle in 22 ppt ASW to remove any excess scum. After 24 hr excess water

was gently removed and the remaining sediment was transferred to sealed bottles and kept at 4°C until needed.

3.2.2. Plastic exposure to Hediste diversicolor

In order to test the hypothesis that microfibers exert a greater toxicity response than beads, exposure experiments were conducted using polyamide (Nylon 6'6) of the following four shape treatments: (1) 23 x 100 µm fibres; (2) 23 x 500 µm fibres (*Barnet Europe*); (3) 23 x 1000 µm fibres (*Barnet Europe*); (4) 10 - 40 µm beads (*Goodfellow*) (fig. 3.1). A treatment with no plastics added to the sediment was also included as a control. The small 23 x 100 µm fibres were created in the laboratory using the developed fibre preparation method by Cole, (2016). Nylon 6'6 thread was aligned around a spool, coated in a water-soluble freezing agent, frozen and cut into ~3 cm sections. Ensuring fibres were aligned, these sections were then bound together into compact blocks using the freezing agent and left to freeze. A cryogenic microtome was used to cut the frozen blocks to the predetermined length of 100 µm. All plastics were fluorescently dyed with Nile Red to allow them to be identified and analysed post-ingestion using a fluorescent microscope. Five millilitres of nile red stock solution (100 mg of nile red in 200 ml acetone) was carefully added to 50 ml falcon tubes each containing one of the plastic types. The tubes were vortexed and left to stand for 10 minutes. The microplastics were then vacuum filtered through 10 µm polycarbonate membrane filters and rinsed with acetone and MilliQ to remove any excess dye. The fluorescently dyed microplastics where then stored in the dark until use.

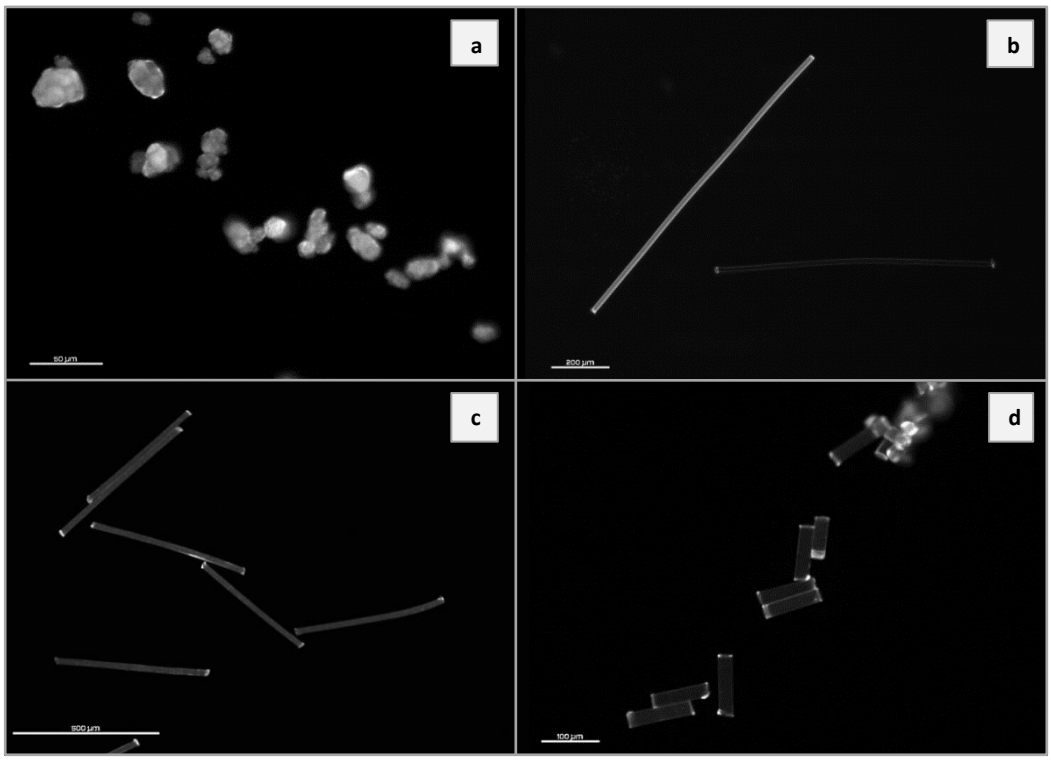

Figure 3.1: Nile Red dyed experimental polyamide (Nylon 6'6) microplastics (a) 10 – 40 µm beads (b) 23 x 1000 µm fibres (c) 23 x 500 µm fibres (d) 23 x 100 µm fibres.

Thirty replicates for each treatment were assembled by adding 25 ml of pre-prepared sediment to 50 ml falcon tubes (n= 150) and microplastics of each treatment at a concentration of 1,359 plastics/ mL (33,976 individual plastics/ replicate). Using microplastic weight as a measure of concentration was deemed inappropriate for this study as the treatments used plastics of different sizes, and therefore the number of plastic particles across treatments would greatly vary based on weight. Thus, the concentration of plastics was determined by calculating the number of plastic particles in 0.01% of the heaviest plastic (23 x 1000 µm fibres) to sediment wet weight (g). This number of plastic particles was then used across all treatments. Although 1,359 plastics/ mL of sediment is still a high concentration compared to that in the marine environment, 0.01% microplastic to sediment weight many times lower than what has been used in previous studies. For example, Wright, et al., (2013a) used concentrations of 0.5, 1 and 5% and Browne et al., (2013) used 5% microplastics when assessing impacts of microplastics in the lugworm, *A. marina*. Once the microplastics were added a sediment: plastic homogenizer, made from a hand – held electric drill

fitted with a small whisk attachment, was used to ensure plastic particles were evenly distributed though-out the sediment. Twenty-five ml of 22 ppt ASW was then gently added to each falcon tube. A hole in each falcon tube lid allowed access for airlines to gently aerate the experimental setup (as shown in fig. 3.2 a, b). The experimental set-up was then left for 24hr to acclimatize in a 15 °C control room.

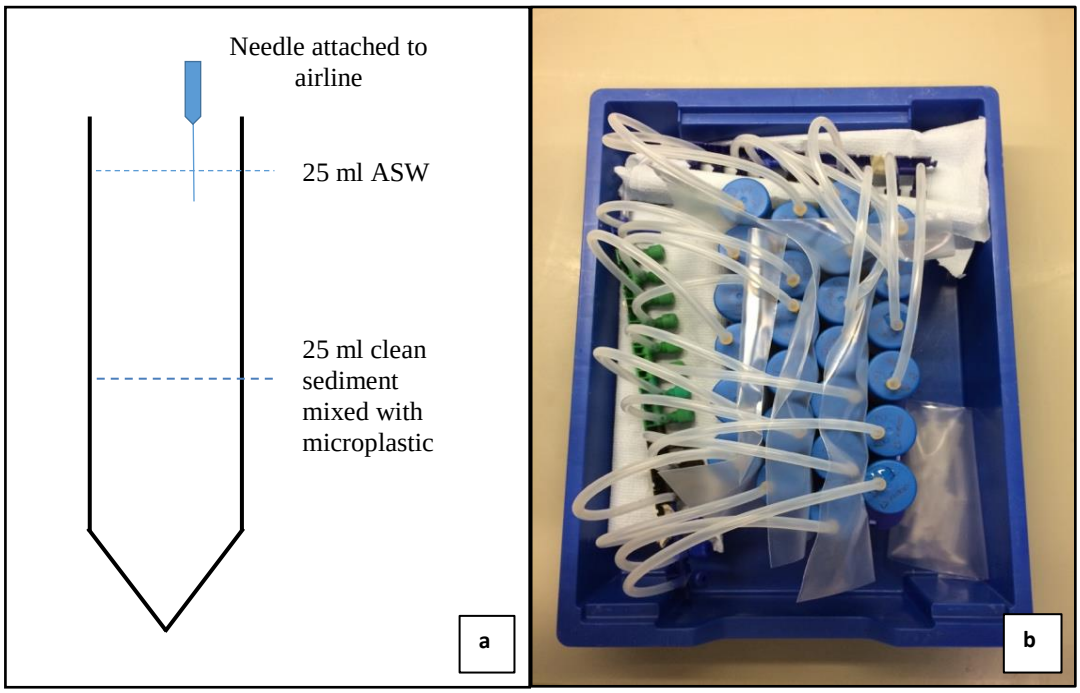

Figure 3.2: *H. diversicolor* plastic exposure experimental set-up. (a) Individual falcon tube set-up. (b) Displaying set-up of 1 out of 5 trays containing exposure containers with equal number replicates of each treatment in every tray.

Prior to the start of the exposure, *H. diversicolor* individuals were transferred into clean glass holding tanks containing 22 ppt ASW at 15 °C for 24 hrs to void their gut content. After this depuration period the *H. diversicolor* individuals (n = 300) were randomly pooled into pairs and added to each replicate (i.e. two worms per tube; n = 150). Experimental conditions were measured at the beginning of the exposure (*Sal: 22.0 ppt; ASW pH: 7.83; Sediment pH: 7.7; DO: 98.0%; Temp: 16.9*) using a Mettler Toledo SevenGo Meter with InLab 738 probe for DO, a Mettler Toledo SevenGo Duo Meter with InLab Expert Pro prode for salinity, a InLab Expert Pro probe for ASW pH and temperature, and InLab Solids probe for

sediment pH. After 48 hrs worms where removed from the sediment, washed in 22 ppt ASW, pat dried and wet weight was obtained.

Ten replicates of each treatment (n = 50) where then used for each of the following categories of ecotoxicological endpoints: (1) Assessment of immune function (phagocytosis and neutral red retention (NNR)); (2) Assessment of oxidative stress (SOD and TBARs); (3) Assessment of quantities of plastic ingested. Haemolymph for phagocytosis and NRR were taken immediately after the exposure period (see methods below), whereas samples intended for assessment of oxidative stress and plastic ingestion where snap frozen using liquid nitrogen immediately after being removed from the sediment and kept at -25° C for later analysis.

3.2.3. Digestion and analysis of ingested plastic

In order to quantify the number of plastics ingested by *H. diversicolor* a known rapid digestion technique using a 10 % potassium hydroxide (KOH) solution was utilized (see: Rochman et al., 2015; Dehaut et al., 2016). Each replicate containing 2 *H. diversicolor* individuals where digested in 50 ml of 10% KOH in a 60°C oven for 12 hrs. The digested contents were then vacuum filtered through 10 µm Cyclopore® Track Etched membrane filers. Filters were then dried in 60 °C oven for 1 hr. Each filter was visually analysed under the fluorescent - coupled microscope Olympus SZX16 (515 – 560 nm) and the number of plastics ingested quantified. Number of ingested plastics particles where then calculated per *H. diversicolor* individual. Procedural blanks were not used for this experiment since the use of fluorescently dyed microplastics in this experiment removed the issue of laboratory-based contamination influencing the results.

3.2.4. Phagocytosis and Lysosomal Membrane Stability

Ten replicates, each containing two *H. diversicolor* individuals, from each treatment (a total of 50 replicates) were used to measure the phagocytic activity and NRR in *H. diversicolor*. Using a fine needle, 50 µl of worm haemolymph was taken from each worm, transferred into 2.5 ml Eppendorf and kept on ice. The haemolymph samples from the 2 worms in each replicate were randomly

allocated to either phagocytosis or NRR assays. At no point was the haemolymph of 2 worms mixed as this prevented a false measure of a phagocytosis triggered by the coelomocytes engulfing each other's cells.

The phagocytosis of fluorescently FITC-labelled Zymosan particles by worm coelomocytes was used to assess the immune function of *H. diversicolor* (*as in*: Anderson & Mora, 1995). Zymosan particles (*Sigma-Aldrich*) where fluorescently dyed with fluorescein isothiocyanate (FITC) by mixing 1 g of Zymosan with 50 ml FITC solution (5 mg in 50 ml carbonate buffer). Excess FITC was then removed through repeated rinsing steps. The FITC-labelled Zymosan was then re-suspended in phosphate-buffered saline (PBS) at a concertation of 5 x 10^8 particles/ ml. Within a 96 well plate, 50 µl/ well of haemolymph (n = 50) was incubated with the FTIC- labelled Zymosan particles for 30 mins. Samples were then quenched with trypan blue which only quenches the surface flourescence so only the internalized phagocytosed particles are measured. The plate was left to incubate for 10 minites and then the fluorescence was measured (excitation = 495 nm/ emission = 525 nm) using a SpectraMax M5 spectrophotometer. A standard curve consisting of a serial dilution of a known concentration of FITC-labelled Zymosan (5 x 10^8 particles/ ml) and PBS was produced. This standard curve was used to quantify the number of phagocytosed Zymosan particles. The protein content of samples was then measured using Brafords Protein assay to standardize number of phagocytosed FITC-labelled Zymosan particles by protein content of each worm (phagocytosed FITC-labelled Zymosan/ µg protein).

Prior to the main exposure experiment, the fluorescence phagocytosis assay was first tested using two positive controls: (1) 3 x pokes from a needle; (2) Injection of lipopolysaccharide (LPS) used as a proinflammatory stimulus. These positive control tests were conducted to confirm if the assays were functioning properly and to test whether the piercing of the worm's epidermis by a needle induces a phagocytic response when bloods are taken. The first positive control group (n= 3) had 50 µl of LPS injected (n= 3) through three injections 1 hr prior haemolymph extraction. The second positive control group (n=3) were poked 3 times/ worm with a needle 10 min prior haemolymph extraction. The phagocytic activity was then measured using the above detailed fluorescent phagocytosis assay.

The uptake and retention of neutral red dye by coelomocytes was used to measure lysosomal membrane stability in *H. diversicolor* as it has previously been shown as a good indicator of overall cell health which correlates with growth and fitness (Lowe et al., 1995). Neutral red retention was quantified using the methodologies of Cajaraville et al., (1996). Worm haemolymph (200 µl/ well) was left to incubate in a poly-L-lysine treated 96 well plate to allow for cells to adhere to the wells. After a 1 hr incubation, non-adhered cells were removed. Two-hundred microliters of 0.2% neutral red working solution (0.058 mg neutral red / mL PBS) was then added to the wells to incubate with adhered cell for 3 hrs. Wells were then rinsed to remove excess dye and 200 µl of acidified ethanol was added to break down cellular membranes and release absorbed dye. The optical density (540 nm) was then measured using Infinite M200 PRO. A standard curve consisting of a serial dilution of neutral red dye and PBS was produced. This standard curve was used to quantify the amount of neutral red dye retained by lysosomal membranes. The protein content of samples was then measured using Brafords Protein assay and used to standardize the amount of neutral red dye by the protein content of each worm (neutral red dye µg/ µg protein).

3.2.5. Oxidative Stress

Ten replicates from each treatment (n= 50) were utilised for SOD and TBAR assays in *H. diversicolor*. Each replicate, containing two *H. diversicolor* were diluted with PBS to establish an equal concentration of biomass and were homogenized into a smooth liquid. This worm homogenate was then split between 2 biological assays measuring oxidative stress: SOD and TBARS.

Superoxide dismutase (SOD) is an antioxidant enzyme which is often used as an indicator of oxidative stress and was quantified using the methodologies of Van der Oost et al., 2005. The SOD assay measures the activity of the antioxidant enzyme via its inhibition of a colourimetric reaction. The assay uses nitrotetrazonlium blue (NBT) and xanthine oxidase (XO) to assess this. XO is used in this assay to generate O_2 radicals. These O_2 radicals react with NBT, creating a colour change as it reduces the amount of the NBT in the wells. In this assay, SOD prevents the O_2 radicals reacting with NBT by catalysing the breakdown of the O_2 radicals and in turn stops the colour change. By using this

colourimetric reaction data (b) and a standard curve consisting of a serial dilution of SOD (S) we can work out the SOD activity in *H. diversicolor* using the following equation.

$$\text{Inhibition} = \frac{b - S}{b}$$

The protein content of the samples was measured using Bradford's Protein assay and used to standardize the amount of SOD inhibition by the protein content of each worm (SOD units/ mg protein).

3.2.6. Thiobarbituric acid reactive substances (TBARs.)

The TBARs assay was used to measure the lipid peroxidation, the oxidative degradation of fats, of cell membranes in *H. diversicolor* by measuring levels of maldionaldehyde (MDA), a by-product of lipid peroxidation, through a colourimetric reaction. In 1.5 ml eppendorfs each sample was created by adding 150 µl mix of PBS and ethylenediaminetetraacetic acid, 20 µl butylated hydroxytoluene, 75 µl trichloroacetic acid, 50 µl thiobarbituric acid and 50µl of worm homogenate. This mix was then vortexed and incubated for 1 hr at 60° C. After incubation samples were centrifuged for 7 minutes at 13000 rpm and 100µl of supernatant added to a 98-well plate in triplicate along with 100µl of PBS and ethylenediaminetetraacetic acid. A prepared standard curve consisting of PBS, ethylenediaminetetraacetic acid, tetraethoxypropane and ethanol, was also added to the well plate. The optical density (530 nm) of the plate was then measured using Infinite M200 PRO. Data collected on the level of lipid peroxidation will indicate if the levels of oxidative stress are significant enough to cause cellular damage. The protein content of the samples was measured using the Bradford's Protein assay and used to standardize the amount of TBARs activity (TBAR activity/ mg protein).

3.2.7. Statistical analysis

Differences in the wet weight of the worms across the treatments were assessed. The data were analysed using the statistical package MINITAB 16. All data were first tested for normality using the Kolmogorov-Smirnov test. If normal, the data were analysed using a one-way ANOVA. If non-normally distributed, data were log transformed and retested for normality. If still non-normal the non-parametric analysis Kruskal-Wallis test was utilized.

3.3. Results

3.3.1. Ingested plastic

No significant differences in worm wet weight was found across the treatments (One-way ANOVA, $F_{4, 284} = 0.51$, $p = 0.728$). Therefore, it can be deduced that worm size did not affect the amount of plastic ingested. No plastics were found in control treatments however ingested plastic was found across each experimental treatment. There was a significant effect of plastic shape on the quantity of plastic ingested/ *H. diversicolor* individual with 10 – 40 µm beads having the greatest quantity ingested/ individual (One-way ANOVA, $F_{4,46} = 16.52$, $p = <0.000$). In contrast, there was no significant effect of fibre size on the quantity of plastic ingested (Tukey post hoc, $p = <0.05$). The average amount of plastic ingested per *H. diversicolor* individual was: 39.2 particles (10 - 40 µm beads); 14.75 particles (23 x 100 µm fibres); 9.20 particles (23 x 500 µm fibres); 5.25 particles (23 X 100 µm fibres) (fig. 3.3).

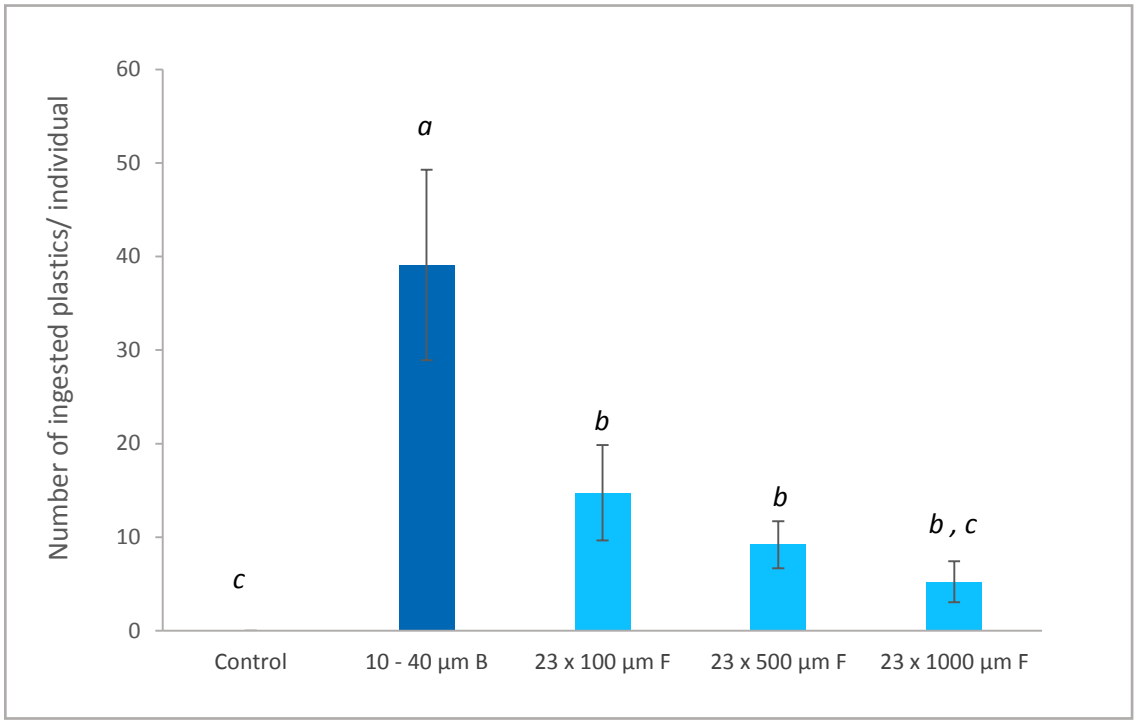

Figure 3.3: Number of microplastics ingested per *H. diversicolor* individual during 48 hr exposure *(B= beads; F= fibres).*

3.3.2. Phagocytosis and neutral red retention

Worms in the control treatments with no microplastics added had a phagocytosis rate of 775.23 ± 124.88 zymosan particles/ µg protein. The positive control using LPS showed a positive response with a significant increase in phagocytosis from the control treatment (Mann-Whitney U, W = 37.0; p = 0.0321) which had a phagocytosis rates of 2220.15 ± 514.50. The second positive control, poke, showed no significant difference from the control (Mann-Whitney U = 43.0; p = 0.358) with a phagocytic rate of 1089.84 ± 165.53. Therefore, any effect of collecting worm haemolymph via a needle can be eliminated. There was no effect of microplastic ingestion on immune function, measured as phagocytosis activity, for any of the microplastic treatments (One-way ANOVA, $F_{4,42}$ = 0.66; p = 0.623) with measures of phagocytosis rates (zymosan particles/ µg protein) varying from 1207.123 ± 248.8 to 885.0 ± 206.13 (fig. 3.4).

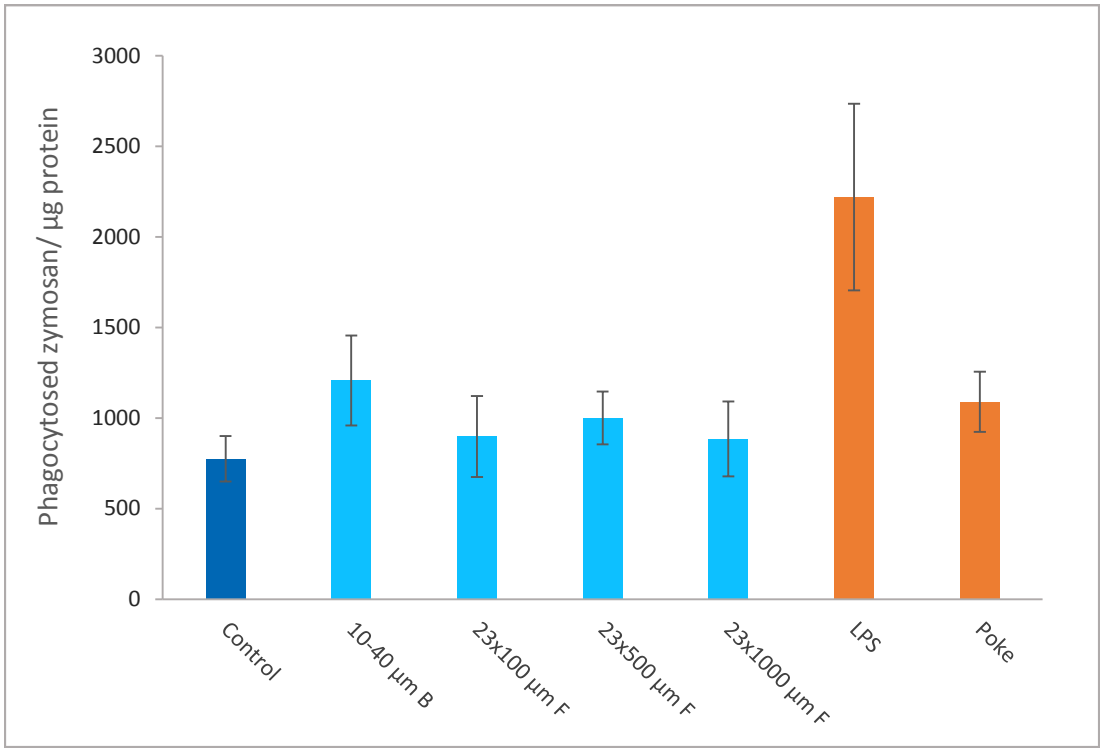

Figure 3.4: Amount of phagocytosed FTIC-labelled zymosan particles by *H. diversicolor* coelomocytes/ µm protein after 48 hr microplastic exposure. (B= beads; F= fibres; LPS & Poke = positive controls).

The amount of neutral red µg/ ml/ µg protein in the control treatment worms was 0.04 ± 0.0. There was no significant effect of microplastic ingestion on neutral red retention for any of the microplastic treatments used (One-way ANOVA, $F_{4, 41}$ = 1.41; p = 0.247). The amount of neutral red µg/ ml/ µg protein for the 10 – 40 µm bead treatment was 0.02 ± 0.01. For the fibre treatments, the amount of neutral red µg/ ml/ µg protein was: 23 x 100 µm fibres = 0.03 ± 0.01; 23 x 500 µm fibres = 0.02 ± 0.01; 23 x 1000 µm fibres = 0.02 ± 0.01 (fig. 3.5).

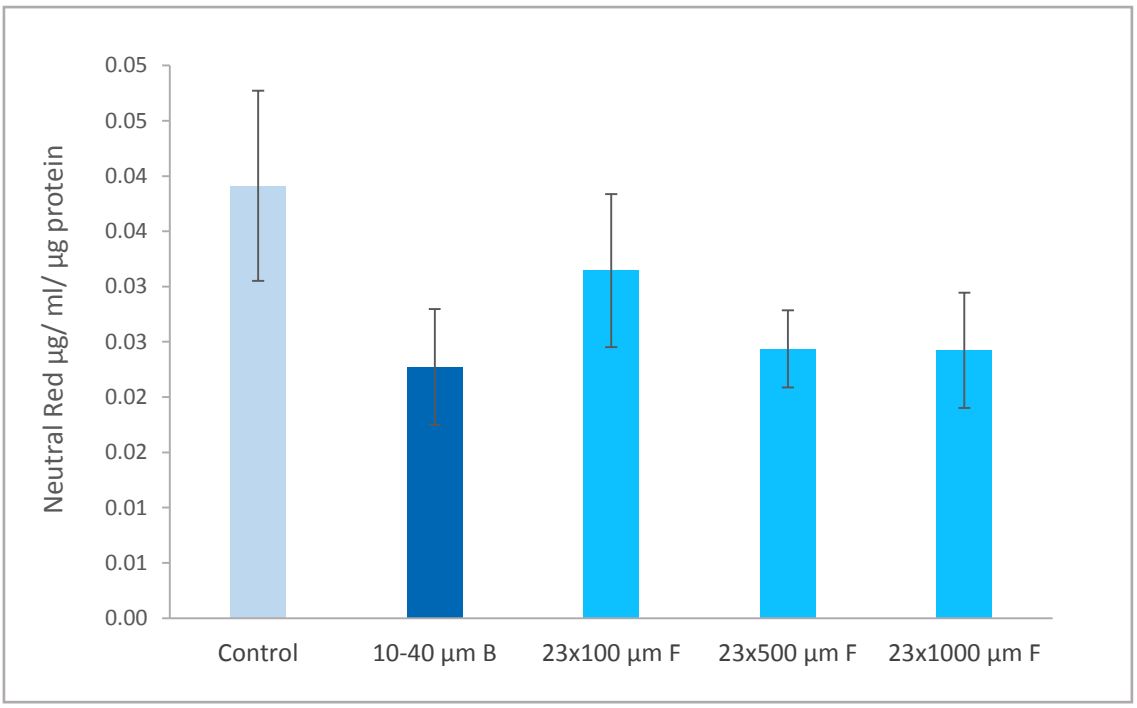

Figure 3.5: Neutral red retention by *H. diversicolor* lysosomal membranes after 48 hr exposure to microplastics *(B= beads; F= fibres).*

3.3.3. Oxidative Stress Assays

The units of SOD activity/ mg protein for the control treatment worms was 0.34 ± 0.04. There was a significant impact of plastic treatment on worm SOD activity (non-parametric analysis Kruskal-Wallis, H (4) = 25.71, p = 0.000). The SOD activity/ mg protein of worms exposed to 10 – 40 µm beads was 0.4 ± 0.05 and were not significantly different to that measured for the control worms (Mann-Whitney U post hoc tests; W = 90.0, p = 0.273). However, there was a significant decrease in SOD activity in worms exposed to all three fibre treatments compared to that of the control (Mann- Whitney U post hoc tests; for 23 x 100 µm fibres vs control W = 133.0, p = <0.001; for 23 x 500 µm fibres W = 141.0, p = <0.001; for 23 x 1000 µm W = 128.0, p = 0.025) and a significant decrease in SOD activity in worms compared to that of the 10 – 40 µm beads (Mann-Whitney U post hoc test; for 23 x 100 µm fibres, W = 134.0, p = <0.001 for 23 x 500 µm fibres, W = 144.0, p = <0.001; for 23 x 1000 µm, W = 135.0, p = 0.005). The units of SOD activity/ mg protein for the worms exposed to the fibre treatments was: 23 x 100

µm fibres = 0.14 ± 0.01; 23 x 500 µm fibres = 0.15 ± 0.01; 23 x 1000 µm fibres = 0.18 ± 0.04.No significant differences in SOD activity was found between fibre treatments (Mann - Whitney U post hoc test; for 23 x 100 µm vs 23 x 500 µm, W = 65.0, *p* = 0.532; for 23 x 100 µm vs 23 x 1000 µm, W = 70.0, *p* = 0.885; for 23 x 500 µm vs 23 x 100 µm, W = 85.0, *p* = 1.00) (fig. 3.6).

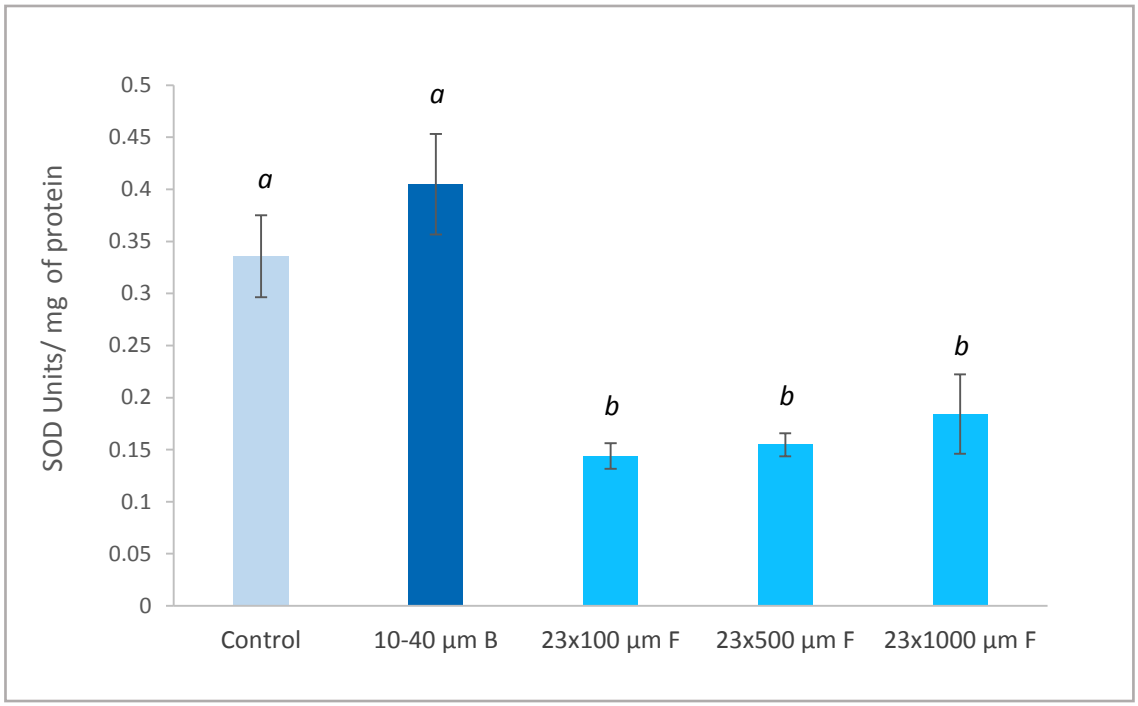

Figure 3.6: Average SOD activity in of *H. diversiolor* specimens' exposure to microplastics (B= beads; F= fibres).

There was a significant effect of treatment on the amount of TBARs in *H. diversicolor* (One-way ANOVA, $F_{4, 39}$ = 18.90, *p* = <0.001) with TBARs values as follows: control = 1.3 ± 0.08; 10 – 40 µm beads = 0.75 ± 0.01; 23 x 100 µm fibres = 1.87 ± 0.16; 23 x 500 µm fibres = 2.04 ± 0.17; 23 x 1000 µm fibres = 1.84 ± 0.17. The amount of TBARs was significantly lower in the 10 – 40 µm bead exposure treatment than that of the control (p = <0.050). The amount of TBARs was significantly greater in worm's exposed to the fibre treatments compared to that of the control (p = <0.05). The amount of TBARs was significantly lower in the 10 – 40 µm bead treatment than that of all fibre treatments (p = <0.050) (fig. 3.7).

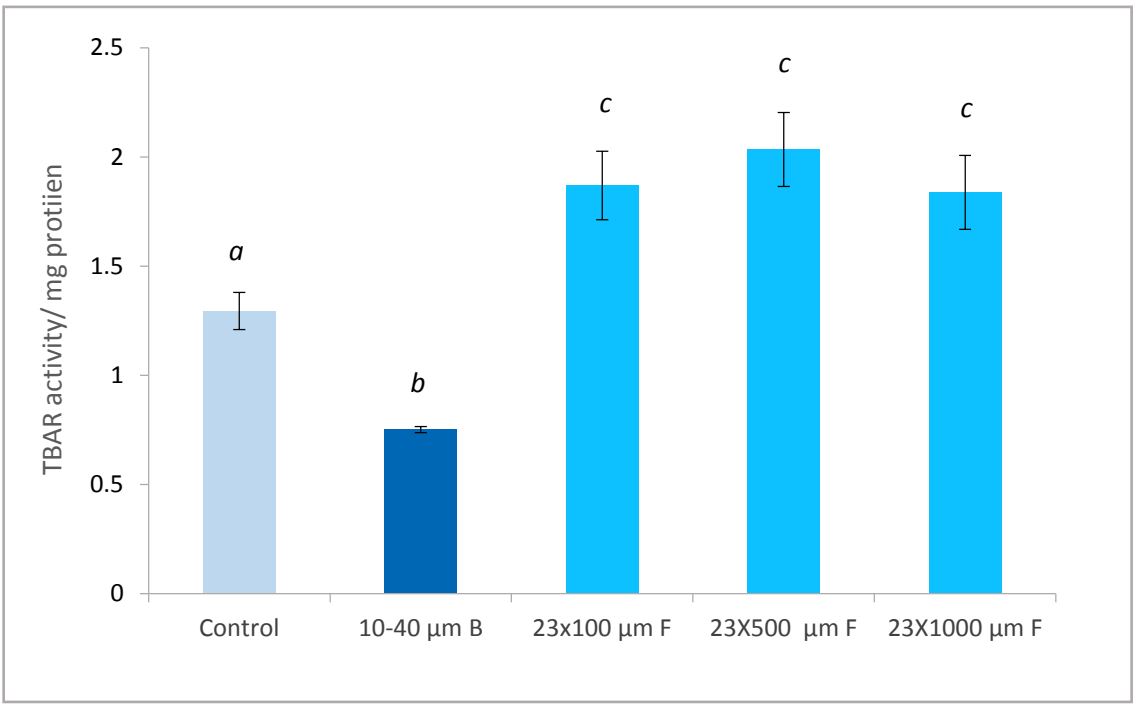

Figure 3.7: Average amount of TBARs in *H. diversicolor* specimens exposed to microplastics (B= beads; F= fibres).

3.4. Discussion

This study has clearly shown that microplastic shape has an important role in the uptake and toxicological effect on the marine polychaete worm, *Hediste diversicolor*. Ingested microplastic was observed in all *H. diversicolor* individuals across every experimental treatment, despite shape or size. There was, however, a clear difference in the quantity of plastic ingested between the bead and fibre treatments with significantly higher quantity of beads consumed (fig. 3.3) suggesting plastic shape plays an important role in its uptake. This is supported by a recent study which compared the uptake and effect of microplastic beads, fragments and fibres in the grass shrimp, *P. pugio*. It found shape to have a significant effect on the number of microplastics ingested with fibres being the least ingested (4.12 ± 6.27 particles/ shrimp), beads being the second most ingested (9.0 ± 13.55) and fragments being the most ingested shape (22.23 ± 9.57) (Gray & Weinstein, 2017). It is possible the complex shape of a fibre makes consumption more challenging than that of beads. However, as shown in chapter

2, fibres are the most commonly reported ingested plastic item in wild populations, including wild populations of *H. diversicolor*. However, this high level of microfiber ingestion could potentially reflect the fact that microplastic fibres are the predominant form in the marine environment. For example, fibres make up 94% of microplastics in Atlantic Ocean sub-surface waters between the Bay of Biscay and Cape Town, South Africa (La Daana et al., 2017), 85% in sediment and bottom waters from the Irish continental shelf (Martin et al., 2017) and 72% in mangrove sediments in Singapore (Nor & Obbard, 2014). Although this high fibre: beads abundance ratio in real-world scenarios are observed, in this study equal numbers of plastic particles were used across the treatments in order to eliminate inconsistency. In addition to this, microplastic fibres observed from the marine environment are often soft and flexible in texture whereas the fibres used in this study where rigid and “rod” like. This could have made the fibre more difficult for the worms to consume than of those found in the marine environment. These fibres were used to test the hypothesis about shape and therefore needed a model fibre that we could produce in large numbers. There was no significant effect of microfiber size on the quantity of plastic ingested. There was, however, a slight trend indicating smaller fibres are more readily ingested by *H. diversicolor* (fig. 3.3). With only three size ranges tested here and all three ingested, an upper limit of ingestible size of fibre could not be determined. Future research should consider a more in-depth analysis of the effects of plastic size on uptake and the complex factors surrounding this.

This study shows a clear impact of microfiber ingestion on oxidative stress that was not observed in response in ingested beads. SOD (superoxide dismutase) activity was significantly lower in *H. diversicolor* individuals exposed to fibre treatments compared to those exposed to the bead and control treatments, with an average 52% decrease in SOD activity in the fibre treatments compared to the control treatment (23 x 100 µm = ~57%; 23 x 500 µm = ~54%; 23 x 1000 µm = ~45%). Corresponding with this decrease there was significant increase in the amount of oxidative damage to lipids, measured as TBARs (thiobarbituric acid reactive substances) in *H. diversicolor* individual’s exposed to the fibre based treatments. Similar results for microbeads were reported in a recent study by Gomiero et al., (2018) in which they compared multiple toxicological effects of ingested virgin micro-polyvinyl chloride beads and benzo (a) pyrene spiked

micro-polyvinyl chloride beads on *H. diversicolor*. They reported no evidence of oxidative stress in these individuals, measured as ROS production, antioxidant catalase activity and lipid peroxidation. This study supports our finding that fibres have the potential to cause greater oxidative stress than that of microplastic beads. There was a 17.6% increase in SOD activity in *H. diverscolor* individuals' exposed to the 10 – 40 µm beads compared to that of the control worms. Whist this was not statistically different to the control worms the increase in SOD activity levels corresponds to a 42.3% decrease in lipid peroxidation (measured as TBARs) in these worms. Combined these results suggest the beads induced a mild oxidative response which the worms have responded to by increasing their expression of SOD, which then is protective against oxidative damage leading to a decrease in lipid peroxidation.

Reactive oxygen species (ROS) are produced during normal cellular metabolism, but can be elevated and induce oxidative stress during an inflammatory response. During inflammation, immune cells, such as mast cells and leukocytes, accumulate at the site of damage which can cause a "respiratory burst". This increase of cellular metabolism increases the amount of ROS produced at the site of damage (Reuter et al., 2010). ROS such as superoxide radicles, hydrogen peroxide, hydroxyl radicals and reactive nitrogen species, can cause cellular damage if not regulated by an individual's biological system. These ROS can cause damage to lipids, proteins and DNA, thus oxidative stress is an essential stress response in organisms, especially in those exposed to changing environmental conditions such as UV exposure, thermal stress, and pollution (Lesser, 2006). The rationale for assessing immune and oxidative stress responses in this study are because fibres, such as asbestos, are well known to induce an inflammatory response (Janssen et al., 1994; Hamilton et al., 1996; Manning et al., 2002). It is, therefore, possible that microplastic fibres could create more damage than beads by inducing a greater inflammatory response and in turn oxidative stress and damage.

The degree of damaged caused by oxidative stress can be dependent on the efficiency of an organism enzymatic defences (Amiard-Triquet et al., 2012). SOD is an antioxidant that breaks down O_2 free radicals into H_2O_2 so they are no longer harmful to cells. It is often classed as a primary defence and is present in almost

all oxygen respiring organisms (Yu, 1994). In this current study, a reduction in SOD activity in *H. diversicolor* individuals exposed to fibres suggests that the ingestion of microplastic fibres reduces the capacity of *H. diversicolor* to deal with oxidative stress, potentially due to the reduction of SOD activity caused by either direct damage to the SOD protein (proteolysis), and therefore consequently the enzyme is unable to work effectively enough to regulate the free radicals, or through the potential inhibition of SOD production itself (as suggested by Browne et al., 2013). This is reflected in the fact there was an increase in cellular damage (lipid peroxidation) in these individuals which was measured as the increase of TBARs, a by-product of lipid peroxidation. This suggests that the oxidative stress levels in *H. diversicolor* exposed to fibres were significant enough to measure damage to cellular components. The potential for microplastics to affect the capability of organisms to deal with oxidative stress has been shown before. For example, Browne et al., (2013) reported the lugworm, *A. marina*, had a >30 % reduced capacity to deal with oxidative stress when exposure to sediments spiked with 230 µm polyvinyl chloride granules.

During the current study, no significant difference in the number of phagocytosed FTIC-labelled zymosan particles engulfed by *H. diversicolor* coelomocytes between treatments was observed. This indicates that neither the ingestion of microplastic beads or fibres stimulates a phagocytic response in this species of polychaete worm. However, evidence of increased immune function in worms has been shown before in previous studies. For example, Wright et al, (2013a) found lugworm (*A. marina)* display an increase in phagocytic activity when exposed to unplasticized polyvinyl chloride beads (230 µm) as well as a reduction in feeding activity and energy reserves compared to that of non-exposed worms. In another example, Gomiero et al., (2018) report *H. diversicolor* individuals exposed to virgin polyvinyl chloride beads displayed a 20 – 25% increase in immune system activity, measured as phagocytosis. Although non-significant, all exposure treatments showed slightly higher levels of phagocytosed zymosan compared to the control treatment with the highest levels exhibited in the 10 - 40 µm bead treatments. This could indicate that beads may have a greater impact on an individual's health than fibres, this could be explained by the greater amounts of beads ingested compared to the fibres in this study. Due to the fact that during inflammation there is an increase in immune-based cells, it is

surprising that in this study no increase in phagocytosis was observed. No significant differences in the amount of retained neural red dye by *H. diversicolor* lysosomal membranes were found, suggesting that the stability of the lysosomal membranes was not compromised by the ingestion of either microplastic beads or fibres in this species. This mirrors what Gomiero et al., (2018) where they found no evidence of reduced lysosomal membrane stability in *H. diversicolor* exposed to virgin polyvinyl chloride beads. However, phagocytosis and lysosomal membrane stability only measure a part of an organism's immune function and therefore further investigation of the impacts of microplastic on an organism's immune system is needed such as DNA damage.

Maintaining homeostasis, especially when dealing with oxidative stress by producing SOD and repairing its consequential damages, is an energetically demanding process (Sokolova et al, 2012). It is possible that the sub-lethal effects caused by ingested microplastics are energetically costly to an organism. As shown in chapter 2, *H. diversicolor* inhabits environments in which they are constantly exposed to microplastics and are able to ingest them, most of which are fibrous in form. This constant exposure potentially means these organisms have to be constantly dealing with cellular damage caused by these plastics. An organism's fitness is directly subjective to the allocation and regulation of energy. Due to the additional cost of maintenance and repair caused by the sub-lethal effects of ingested microplastic less energy may be available for reproduction (Sokolova et al., 2012). For example, Gardon et al., (2018) found polystyrene microbeads (6 -10 µm) significantly affected the pearl oyster, *Pinctada margaritifera*, energy balance and consequently its reproductive capabilities. After 2 months exposed to the polystyrene beads *P. margaritifera* respiration and ingestion rates and ingestion assimilation efficiency where calculated to establish its scope for growth (SFG). SFG is the extra energy available to the organism additional to that for maintenance. The study found that at a concentration of 0.25 µgL−1 and above there was a significant decrease in *P. margaritifera* SFG, however, no change in actual physical growth was observed suggesting the energy needed for growth was obtained elsewhere else which could be explained by the significant reduction in gametogenesis in the oysters exposed to the polystyrene beads. However, the study also found *P. margaritifera* had increased energetic costs from just being exposed to bead, therefore it is possible fibres

could create a likely additional energetic demand. In addition to this, it is possible that fibres could take up more room in the gut of an organism and in turn possibly reduce the energy intake of organisms. This, alongside the additional energy cost of maintenance, could detrimentally impact an organism's fitness and potentially the health of a population as a whole.

3.5. Conclusion

This study clearly shows ingested microplastic fibres have a greater toxicity than that of beads. We report that, unlike beads, ingested fibres induce a greater oxidative stress response and consequently cellular damage in the harbour ragworm *H. diversicolor*. This study is therefore relevant as the majority of plastic reported in benthic environments and in the gut of benthic organisms is fibrous in form and thus provides an ecologically important insight into the effects of microplastic ingestion in the marine benthic environment.

Chapter 4:

Fitness effects of microplastic ingestion in the marine polychaete worm *Ophryotrocha labronica*.

4.1. Introduction

A challenge in ecotoxicology is the translation of individual-level effects of exposure, as measured in laboratory tests, into population-level effects that have more ecological relevance. If the fitness (defined by Lincoln et al., (1998) as "a measure of the contribution of a given genotype to the subsequent generation relative to that of other genotypes") of individuals is compromised it could ultimately impact the species at the population-level, therefore measuring how a stressor affects the fitness of an individual could give an indication of its effects on a wider scale. Currently, the majority of microplastic ingestion research concentrates on individual-level impacts with a focus on cellular and sub-cellular effects. However, in order to develop effective management of plastic pollution and fully assess its risk to biota, it is essential to understand the potential impacts of plastics across all ecological levels (Galloway et al., 2017a).

To maintain good health all organisms must balance their energy input and output. When this balance is disturbed, for example by a change in feeding or by increased cellular damage from a stressor, such as ingested plastics, an organism's energy allocation must shift to compensate for such change (Amiard-Triquet et al., 2012). Food quality and quantity are considered as two of the most important ecological factors in marine invertebrate population dynamics (Prevedelli & Vandini, 1998) as fluctuations in feeding can have knock-on effects on an individual's fitness (Murphy & Quinn, 2018). There is now an increasing amount of scientific literature reporting changes in feeding activity across different organisms when exposed to microplastics (Besseling et al., 2013; Browne et al., 2013; Wright et al., 2013a; Green et al., 2016). In one example, Cole et al., (2015), exposed the copepod, *C. helgolandicus* to algae at 250 µgC L^{-1} and 20 µm polystyrene beads at 75/ mL^{-1}. After 24 hrs they found *C. hegolandicus* to ingest 11% fewer algae and 40% less carbon biomass. In another study, when

exposed to high levels of polyvinyl chloride at 2% microplastic of sediment wet weight, *A. marina* has significantly reduced feeding rates in the form of faecal casts (Green et al., 2016). There are, however, a number of studies that show no changes in feeding rate in the presence of microplastics (Cole & Galloway, 2015; Korez et al., 2017; Jemec Kokalj et al., 2018; Weber et al., 2018). Such conflicting results, however, could possibly be explained by differences in species and their associated feeding modes, gut morphology and the plastic type, shape, size and relative concentrations used. A reduction in the amount of energy an organism can consume could ultimately lead to overall energy loss and consequently affect the amount of energy available for reproduction. For example, although, after a 9-day exposure to microplastic, Cole et al., (2015) found no significant difference in respiration, mortality and hatching rate in *C. helgolandicus*, there was however a significant effect of microplastic treatment on reproduction with exposed *C. helgolandicus* producing significantly smaller eggs.

Impacts such as cellular damage means more energy needs to be spent on repair which consequently may reduce the amount of energy available for reproduction. As shown in chapter 3 and other studies, ingested microplastics can have harmful effects on benthic dwelling worms. For example evidence of inflammation (Wright, et al., 2013a) and increased susceptibility to oxidative stress (Browne et al., 2013) in *A. marina* when exposed to micro-sized plastics. Such stress and potential damage are energetically expensive to repair.

Ophryotrocha labronica is a small species of iteroparous polychaete worm that inhabits harbour and port like environments (Prevedelli & Vandini, 1998). This species is an ideal test organism for laboratory experiments as it is cheap to cultivate, its small size (maximum 4.5 mm) allows for space saving experiments and is easily transported, it thrives in high populated cultures, they reproduce all year round and produce many offspring over a short period of time (Åkesson, 1970). This species has also been used in many ecotoxicological studies (Saliba & Ahsanullah, 1973). During preliminary microplastic exposures conducted at Exeter University, *O. labroncia* was reported to ingest 10 – 40 µm polyamide beads (Lewis, 2017: *in conversation*). The objective of this chapter was to assess if ingested microplastics impact *O. labronica* feeding rate and consequently their reproductive output.

4.2. Methods

4.2.1. Animal husbandry

Ophryotrocha labronica were chosen as the study species rather than *Hediste diversicolor* as they are known to reproduce well under laboratory condition all year round and the individual sexes identified easily compared to that of *H. diversicolor* individuals. It is not possible to rear *H. diversicolor* through gametogenesis to reproduction under laboratory conditions due to the semelparous reproductive strategy of this species. Adults switch to reproductive rather than feeding mode anywhere between the age of 1 and 7 years and then cease feeding when producing gametes.

O. labronica were originally collected from Grado Harbour, Italy and transported to Exeter University aquarium in March 2016 in order to start a laboratory-based culture. In March 2017 ~ 20 males were added to the culture to increase the population's numbers. *O. labronica* where kept in 500 ml glass dishes with 450 ml 35 ppt ASW in a 20˚C control room. The cultures were water changed and fed ~5 ml of homogenised spinach once a week.

4.2.2. Plastic Exposure

Prior to the exposure, *O. labronica* individuals were sexed and united to create 1 male to 1 female mating pairs (n= 20). Mating pairs (n= 10) were then each added to individual wells within 2 x 6 well plates along with 10 ml 35 ppt ASW, 200 µl of homogenized spinach and 5000 (500 beads/ ml) 10 – 40 µm nile red labelled polyamide beads. Control worm mating pairs (n = 10) were exposed to the same experimental set-up but without the added polyamide beads. The set-up was kept in the dark in a 20 °C ± 0.06 control room throughout the duration of the exposure. Each mating pair was water changed every 2-days by carefully transferring each pair of worms into a fresh new well with the same plastic, water and spinach concentrations added. Before being placed in a new well, each mating pair was rinsed in 35 ppt ASW to prevent the transfer of plastics into the new set-up (fig. 4.1). Water changes were conducted a total of 25 times over the period of the experimental exposure. Despite our finding from chapter 2 and 3 beads were

used during *O. labronica* exposure. The justification for the use of beads instead of fibres where for two reasons. Firstly, preliminary exposure at Exeter University showed the ingestion of these beads by *O. laborinca*. Secondly, as this species of worm are very small and therefore it would have been unrealistic to of fed it fibres as they would have been too large for them to consume.

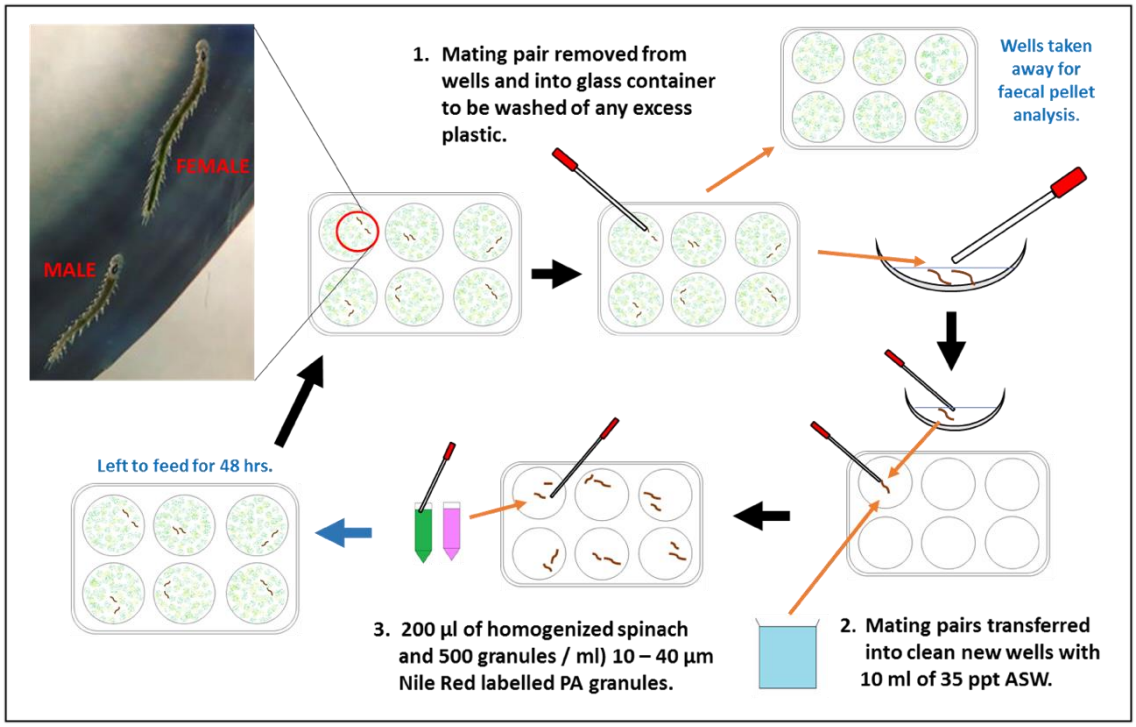

Figure 4.1: Diagram showing the process of *O. labronica* experimental set-up water changes which took place every 48 hrs and conducted a total of 25 times over the exposure period.

4.2.3. Faecal pellet production

As no reliable assay for feeding rate could be developed faecal pellet production was used instead to measure egestion rather than ingestion. After 7 days' exposure, photos were taken of each well using an Olympus SZX16 microscope. The *O. labronica* faecal pellets from the experimental exposure and controls were then analysed using ImageJ and number of faecal pellets and faecal pellet volume (mm^3) then calculated. A sub-sample of faecal matter from each well was

then observed for the presence 10 – 40 µm PA beads under an Olympus SZX16 florescent microscope. Prior to the start of the experiment, each *O. labronica* individual's segments were counted to ensure no differences in worm size between treatments and therefore eliminate the possibility that worm size had an effect.

4.2.4. Fitness endpoints

The first egg mass a mating pair produced was discarded as they are highly variable in size. Each *O. labronica's* second egg mass was left to develop and hatch to ascertain a measure of juvenile survival. Once hatched, juvenile's abundance was measured for 5 days which was used to calculate juvenile survival rate. Parent worms were left with the egg mass as this species display parental care, as well as to prevent any removal of polyamide beads and juveniles. Mating pairs were then transferred into a fresh experimental set-up under the same conditions to produce their third egg mass in the same method as a water change (see: fig. 4.1). Once the third egg mass was produced it was removed and photographed using a Zeiss Observer Z1 microscope. Using ImageJ the number of eggs per egg mass was counted to ascertain a measure a fecundity and average maximum egg diameter was measured. These eggs were then carefully transferred into Eppendorf's and their protein content measured using the Bradford's Protein Content assay.

4.2.5. Statistical analysis

Data were analysed using the statistical package MINITAB 16. All data were first tested for normality using the Kolmogorov-Smirnov test. If normal, the data were analysed using a two-sample t-test. If not normally distributed, data were log transformed and retested for normality. If still non-normal the non-parametric analysis Mann-Whitney U test was utilized.

4.3. Results

4.3.1. Faecal pellet production

No significant difference in the number of worm segments between treatments was found (*Mann-Whitney U*, W = 579.5, *p* = 0.173; fig. 4.2). Therefore, the effect of worm size on feeding rate can be eliminated from this study.

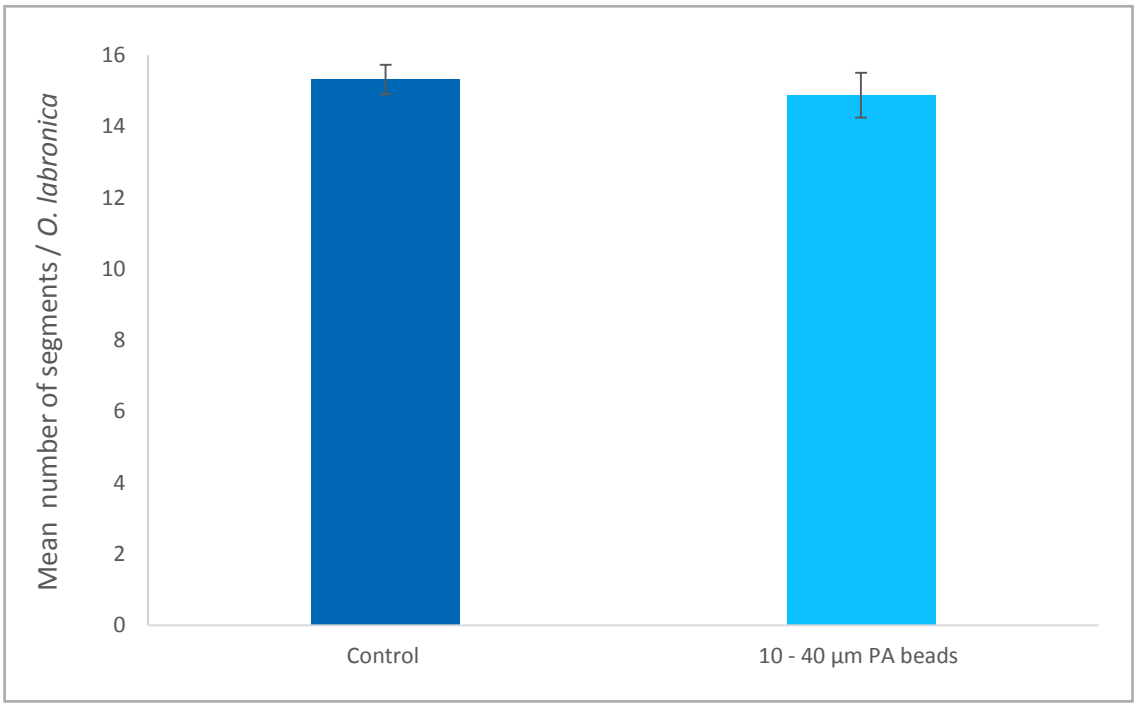

Figure 4.2: Average number of segments per *O. labronica* between control and experimental treatment.

A significant difference in number of faecal pellets was found between the control and exposure treatments (*Two-sample t-test,* t (21) = 2.75, *p* = 0.014; fig. 4.3 a) with the 10 – 40 µm PA bead treatment having the greatest number of faecal pellets (Mean number of faecal pellets/ mating pair: Control 43.45 ± 4.93; 10 – 40 µm PA beads: 70.75 ± 8.60)

There was also a significant difference in faecal matter volume (mm^3) between the exposure and control treatments (*Mann-Whitney U*, W= 548293.0, *p* = 0.021;

fig. 4.3 b), with the 10 – 40 µm PA beads treatment having the greatest volume of faecal matter mm^3 (Mean volume of faecal matter mm^3/ mating pair: Control = 0.082 mm^3 ± 0.02; 10 – 40 µm PA beads = 0.1 mm^3 ± 0.02). The presence of the 10 – 40 µm beads were found in the faecal pellets of the individuals in the exposure treatment (see: fig. 4.4 c-f). No evidence of 10 – 40 µm beads was present in the control worm's faecal pellets (see: fig. 4.4 a-b).

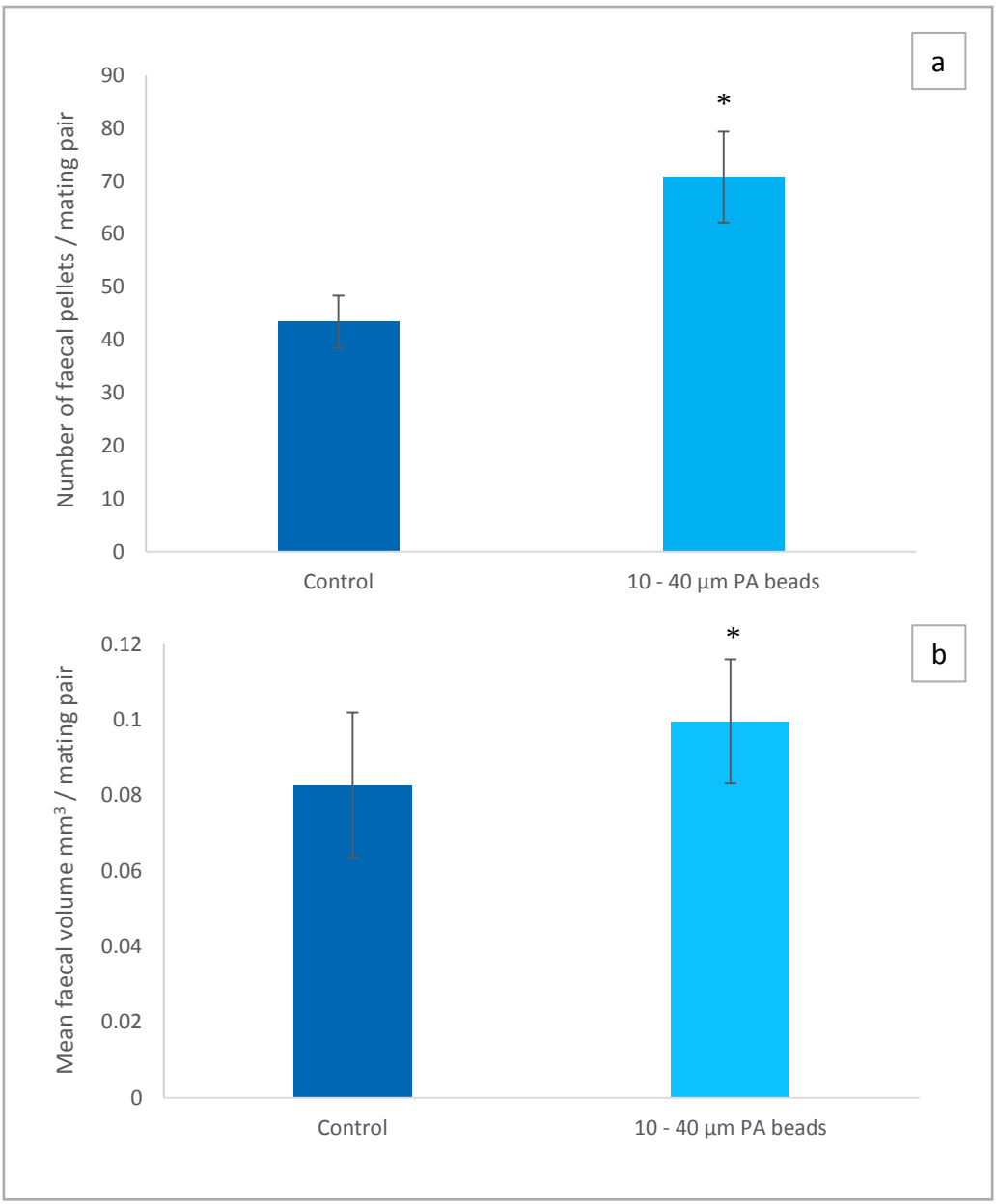

Figure 4.3: (a) Average number of faecal pellets per *O. labronica* mating pair (b) Average volume of faecal matter mm^3 per *O. labronica* mating pair.

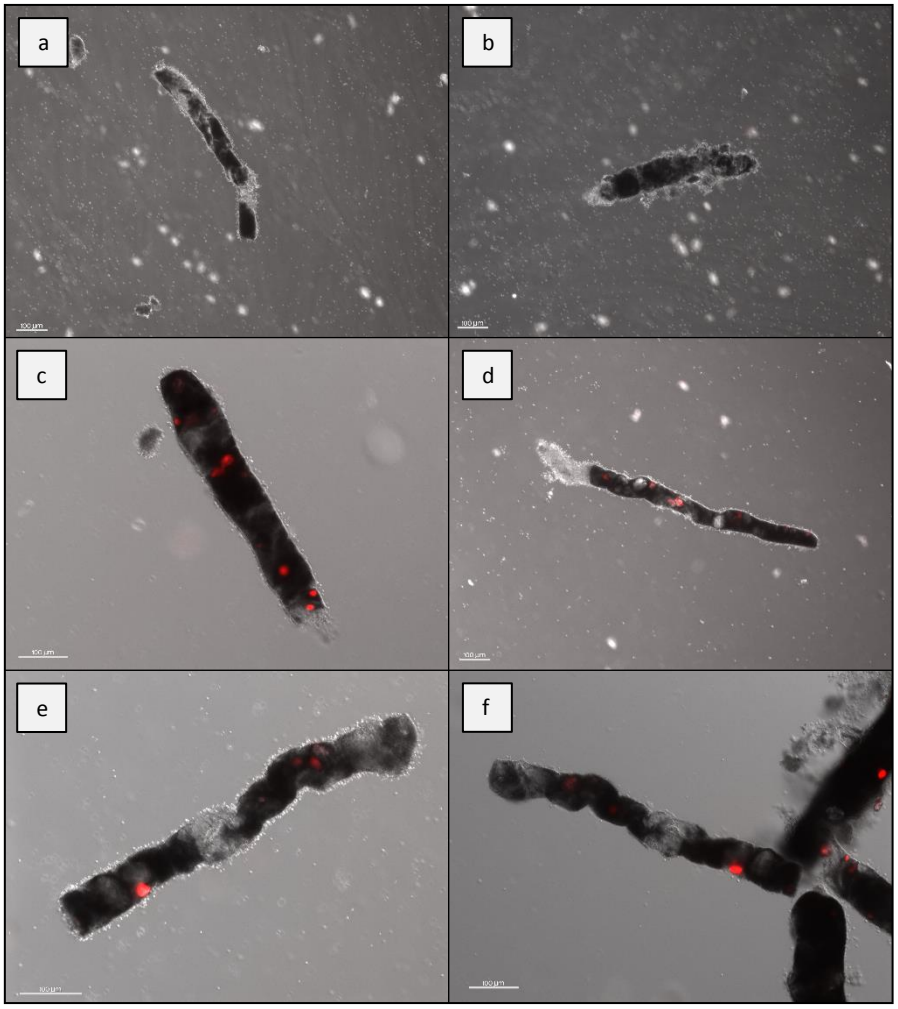

Figure 4.4: (a-b) Faecal pellets from control *O. labronica* with no evidence of 10 - 40 µm PA bead. (c - f) Faecal pellets from *O. labronica* exposed to 10 – 40 µm PA beads (Nile red dyed beads fluorescing red).

4.3.2. Fecundity

In total 10 egg masses (control: n = 5/ 10 – 40 µm PA beads: n = 5) were produced by *O. labronica* and then analysed for the number of eggs/ egg mass. The mean number of eggs/ egg mass were: control = 85 ± 24.72; 10 – 40 µm PA beads = 35.6 ± 9.53. However, there was no significant difference in number of eggs/ egg mass between the control and 10 – 40 µm PA bead treatments (*Mann-Whitney U*, W= 37; *p*= 0.06; fig.4.5).

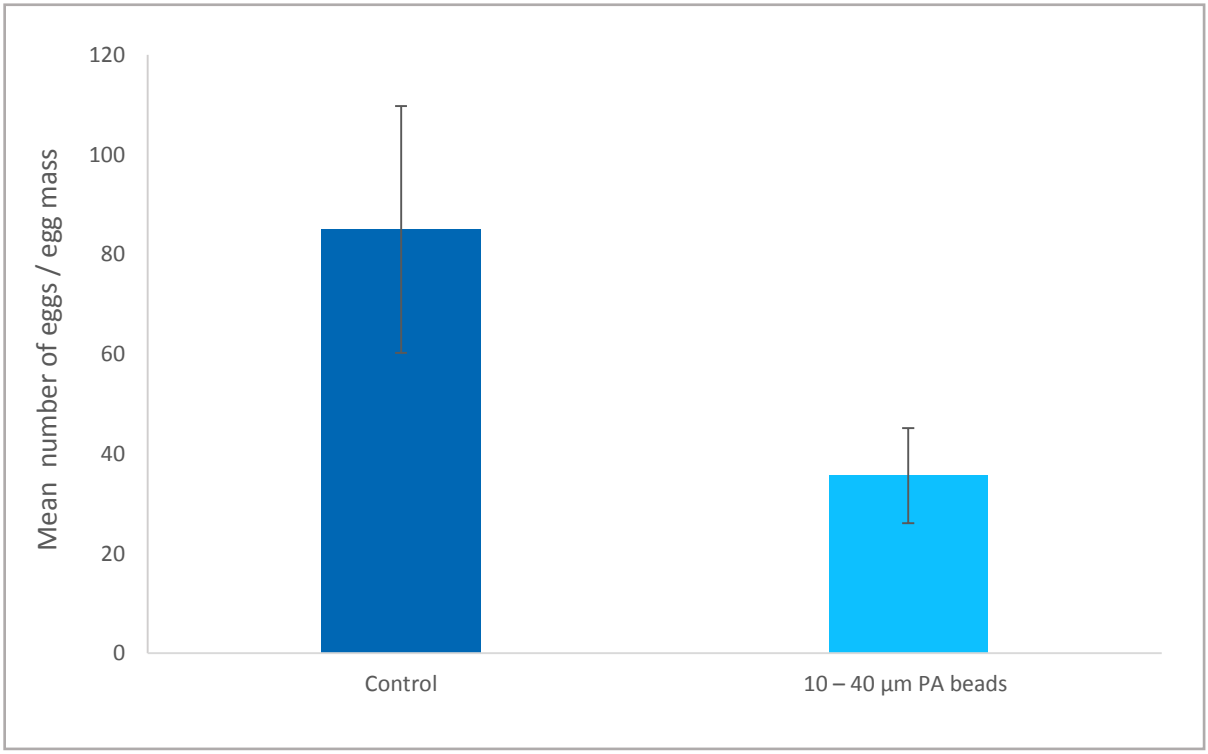

Figure 4.5: Average number of eggs per *O. labronica* egg mass between the control and experimental treatment.

4.3.3. Egg size

There was a significant effect of treatment on egg size between the control and 10 – 40 µm PA bead treatments (*Two-sample t-test,* t (8) = -5.89, *p* =<0.001; fig. 4.6) with the *O. labronica* exposed to 10 – 40 µm PA beads having smaller eggs (Mean diameter of eggs: Control = 178.75 µm ± 6.75; 10 - 40 µm PA beads = 161.27 ± 8.78).

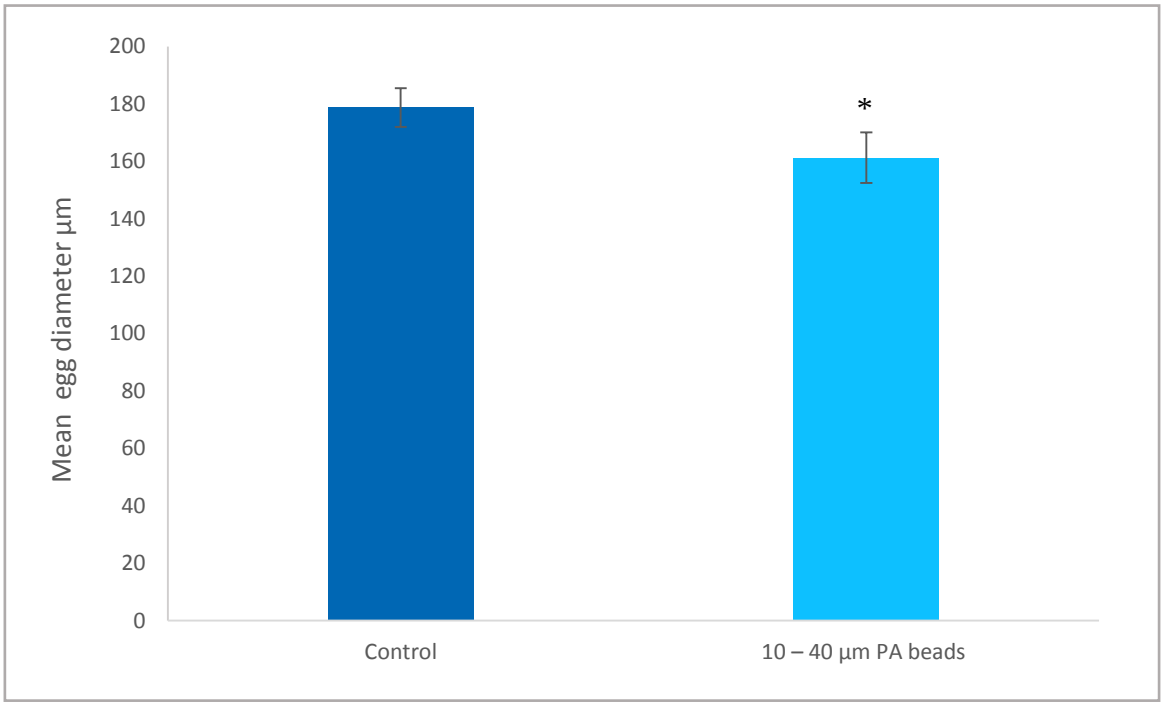

Figure 4.6: Average egg size (maximum diameter µm) between control and experimental treatment.

4.3.4. Egg protein content

There was no significant effect of treatment on egg protein content between the control and 10 - 40 µm PA bead treatments *(Two-sample t-test, t* (7) = 1.59, *p* = 0.187; fig.4.7). Mean egg protein content µg/ egg: Control = 2.28 µg ± 0.37; 10 – 40 µm PA bead = 1.64 µg ± 0.13.

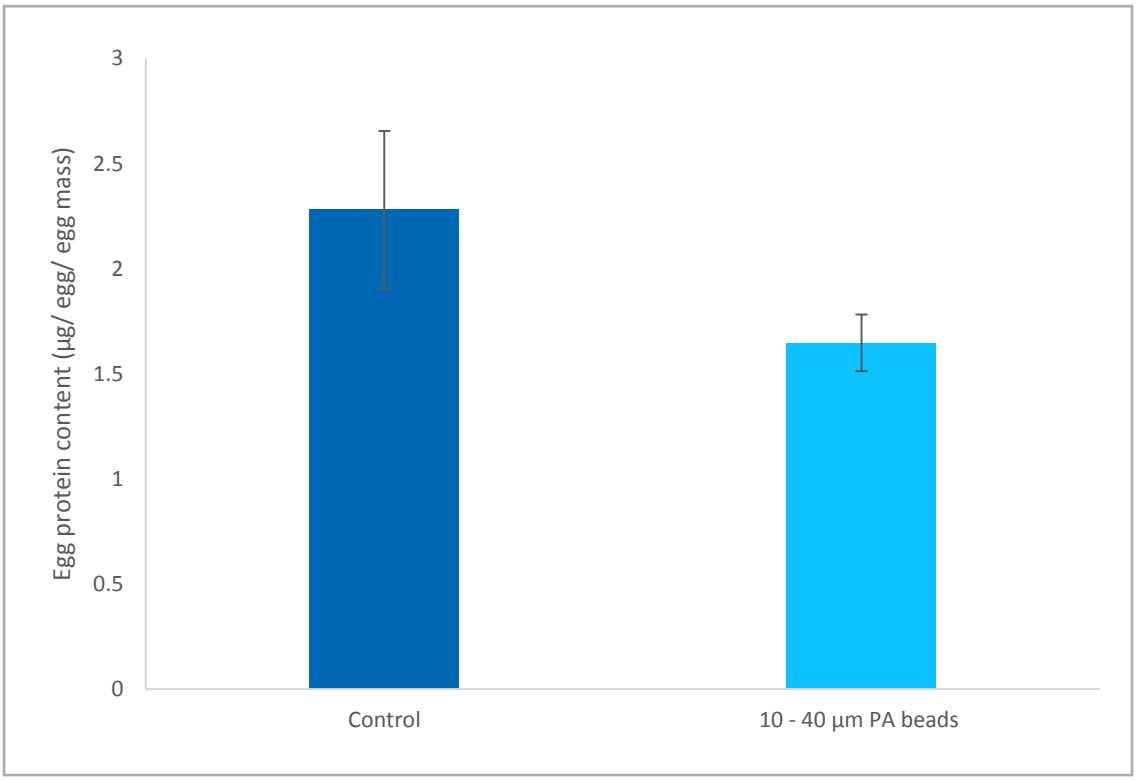

Figure 4.7: Average *O. labronica* egg protein content µg per egg between control and experimental treatment.

4.3.5. Juvenile Survival Rate

There was no effect of treatment on *O. labronica* juvenile survival rate after 5 days (*Two-sample t-test,* t (5) = 1.30, *p* = 0.264; fig. 4.8). Mean *O. labronica* juvenile survival rate: Control = 88.79 % ± 4.86; 10 – 40 µm PA bead = 79.44 % ± 5.32).

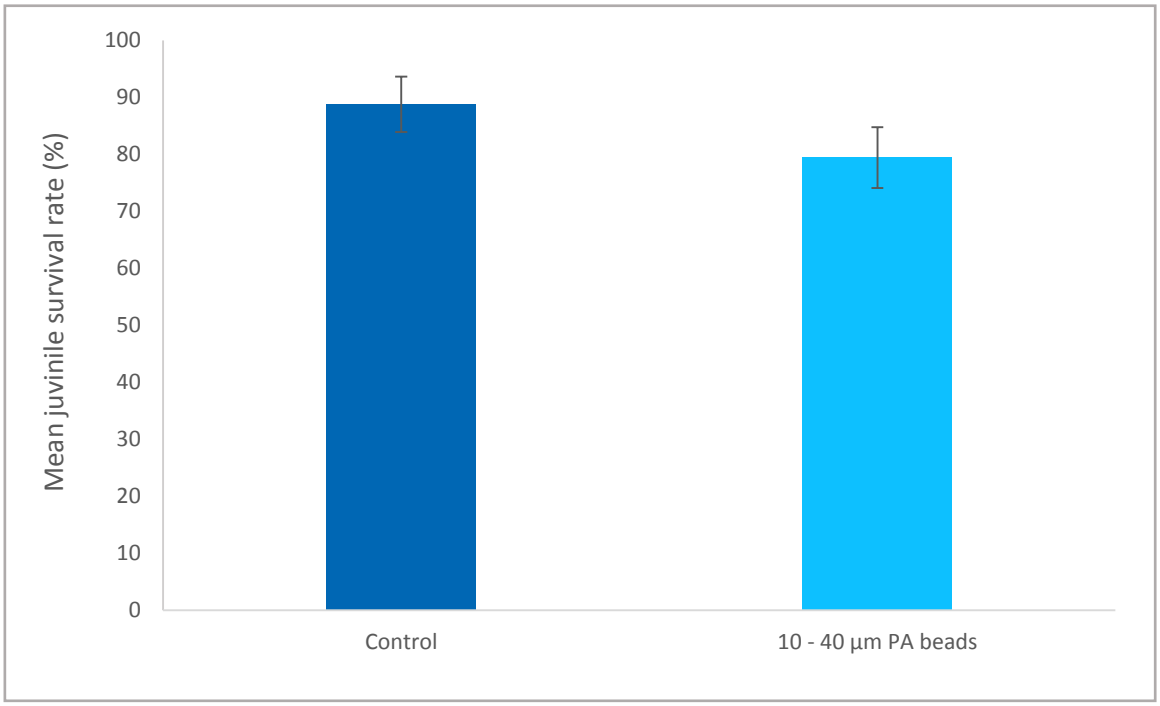

Figure 4.8: Average *O. labronica* juvenile survival rate (%) after 5 days since hatching between control and experimental treatment.

4.4. Discussion

This study found a significant increase in the number of faecal pellets and volume of faecal matter produced by *Ophryotrocha labronica* exposed to 10 – 40 µm PA beads compared to the control worms. However, after digital analysis under an Olympus SZX16 florescent microscope, the 10 – 40 µm PA beads where found incorporated into the faecal pellets of individuals treated to the exposure (fig. 4.4 c-f). Thus, using number of faecal pellets and total faecal matter volume as a proxy for feeding rate in this study is difficult to reliably measure as the 10 – 40

µm PA beads will increase the volume of the faecal pellets and in turn may affect the number produced. Another method, assessing fluctuations in chlorophyll concentration of the food item, spinach, before and after plastic exposure was tested. However, this method proved unsuccessful due to difficulties in separating the faecal pellets from the leftover food.

Although here we cannot directly relate number and volume of fecal pellets to feeding rate, the high percentage increase of the treatment compared to the control (number of fecal pellets = 62.83%/ volume of fecal pellets = 21.95%) suggests an increase not solely due to the incorporation of the beads into the fecal pellets. By assuming all the beads were at the maximum size of 40 µm and spherical in shape, you would need an average 24,470 beads incorporated into the faecal matter per mating pair to account for the difference in faecal pellet volume increase between the control and experimental treatment. Considering only 5000 PA beads were added to each replicate, these plastics could not account for all of the faecal volume and number increase. Although not directly measured this suggests an increase in the feeding rate of *O. labronica* exposed to the 10 – 40 µm PA beads. This potential increase in feeding rate, when exposed to plastic, is the opposite of what most other studies find, in which feeding rate is more than often reduced in the presence of plastic. For example in the freshwater cnidarian, *Hydra attnuta* (Murphy & Quinn, 2018)**,** in the marine copepod, *C. helgolandicus* (Cole et al., 2015) and in the freshwater amphipod *Gammarus fossarum* (Blarer & Burkhardt-Holm, 2016). However, an increase in feeding rate in the presence of plastic and its effect on fitness has been shown before. Sussarellu et al., (2016) found after a 2-month exposure to polystyrene beads (2 - 6µm) at a concertation of 0.023 mg.L $^{-}$1 adult Pacific oysters, *C. gigas*, showed a significant increase in microalgae consumption and absorbance efficiency compared to *C. gigas* that where not exposed to microplastics. This increase in consumption could be due to *C. gigas* compensating for a diet with a reduced nutritional value due to the plastics. This, in turn, had negative impacts on *C. gigas* reproductive health with a 5% decrease in oocyte size, a 38% decrease in oocyte number and a 23% reduction in sperm velocity. They also found a 41% decrease in D-larval yield and an 18% decrease in larval development compared to the offspring of *C. gigas* not exposed to polystyrene

beads. An increased feeding rate may also suggest more energy and time being spent on feeding rather than other important tasks such as reproduction.

Whilst no statistically significant effect of plastic exposure on fecundity, assessed as number of eggs per egg mass, was measured, a an almost 60% decrease in the number of eggs per egg mass was observed and the calculated p-value was close to being significant despite low replication. Only 5 third egg masses per treatment were produced during the 46 days of this experiment, meaning there is very low statistical power to detect a real effect. Due to this high level of variation between samples, it is possible that with more replicates the data might produce a significant result. This suggests the potential for plastics to impact *O. labronica* fecundity by decreased oogenesis, and future works should investigate this more closely as it could have population-level implications. Fecundity is fundamental in life-history theory as it is directly connected to an organism's energy allocation with egg production highly affected by the quantity and quality of food available (Llodra, 2002). Evidence of reduced oogenesis in the presence of microplastics has been reported before, for example, Lei et al., (2018) found when exposed to a 5.0 mg/ m^{-2} of polyethylene, polypropylene, polyamide, polystyrene and polyvinyl chloride, the nematode worm *Caenorhabditis elegans*, has a significant reduction in both its brood size (number of eggs) and embryo number.

This study found a significant effect of treatment on egg size in which *O. labronica* females exposed to 10 – 40 µm PA beads produced smaller eggs (9.8 % decrease in size) than that of the control worms. However, despite the eggs being smaller, there was no significant effect on the energetic investment (protein content) between the exposed and control treatments (fig. 4.7). This was reflected in the fact there was no significant difference in *O. labronica* offspring survival rates between the control and treatment worms as the juveniles were provided with similar reserves (fig. 4.8). This is unexpected as egg size is often thought to be closely related to energetic content and often used as an assessment of material fitness (Moran & McAlister, 2009). However, protein content is only one measure of egg energetic content. Lipid content and carbohydrate content can also be assessed (Moran & McAlister, 2009). Cole et al., (2015) found after a 9-day exposure to 20µm polystyrene beads, the copepod *C. helgolandicus* ingested significantly fewer algae, measured as carbon biomass when exposed to

polystyrene beads compared to unexposed *C. helgolandicus*. Those exposed to polystyrene beads ingested 9.7 µg of carbon/ copepod/ day, whereas those in the control treatment ingested 16.0 µg carbon/ copepod /day. They also found copepods exposed to plastics consumed only the small prey items available (preference for 12.7 -13.7 µm) whereas the unexposed copepods consumed all prey available (11.6 – 17 µm). Consequently, they found exposed copepods produced significant smaller eggs (2.1 % decrease) and eggs had reduced hatching success (reduction of 25.3%). No significant effect of treatment was found on egg production, respiration or mortality. The authors suggest that the reduction in egg size was a result of reduced algae consumption and potentially influenced the reduction in egg carbon biomass which consequently impacted *C. helgolandicus* hatching success.

Egg size is a well establish measure of parental investment and fitness in reproductive ecology (Qian & Chia, 1991) and to the best of our knowledge this is the first evidence of plastics impacting a polychaete worm's fitness by influencing the size of eggs a female produces. Egg size can be influenced by a number of factors such as maternal nutrition, size and age (Moran & McAlister, 2009). Egg size could be an important influential factor in fertilisation success as it affects the effective target size for sperm during fertilisation. A study by Marshall et al., (2000) clearly demonstrated that egg size strongly influences the fertilisation success of the free-spawning intertidal ascidian, *Pyura stolonifera*. They found maternal size greatly influenced egg size with larger females producing larger eggs. They found the eggs of smaller females needed a greater concentration of sperm to reach the maximum level of fertilization success compared to that of the eggs of larger female *P. stolonifera*. In the current study, there was no difference in adult *O. laronica* size between treatments, however, females' exposed to 10 – 40 µm beads produced smaller eggs. Although *O. labronica* displays a different reproductive strategy to *P. stolonifera*, the influence of microplastics on egg size and fertilization success should be considered in future research.

There is an increasing body of research assessing the impacts of microplastic on larval and juvenile organisms. For example, observed growth delays in larval slipper snail, *Crepidula onyx* (Lo & Chan, 2018), and reports of slower

metamorphism and altered plutei development in the ascidian, *Ciona robusta* (Messinetti et al., 2017). However, limited research has been reported on how parental exposure to microplastics impacts the resulting offspring development. We suggest a research focus on the continuous monitoring of plastic exposed organisms over multiple generations, assessing the growth, development, survival and fitness is required to gain a greater understanding of the impacts of microplastic at the population level.

4.5. Conclusion

In conclusion, *O. labronica* adult mating pairs exposed to 10 - 40 µm PA beads produced less offspring and significantly smaller eggs than unexposed mating pairs which ultimately could lead to deleterious impacts at the population level. However, the eggs that were produced had a similar energetic content and in turn, the offspring produced from those eggs had a similar survival rate. Exposed worms also had an increase in faecal pellet production, an increase that couldn't be fully accounted for by the added volume of incorporated plastics. This increase could suggest an increase in feeding rate however further analysis is needed to determine this. . It is possible chronic effects could arise over multiple generations of polychaete worms being exposed to plastics and would make for noteworthy research. When assessing these impacts future research could also assess changes in energy allocation to determine the more consequential impacts of microplastic ingestion.

Chapter 5:

General Discussion

The marine benthic environment is known as a major sink of microplastics, in which both positively and negatively buoyant plastics accumulate. As a result, the infaunal community are likely to come into contact and interact with these debris. However, the impact of plastics on these organisms is still largely unknown. This project aimed to address the question: “what impact does microplastic ingestion have on marine polychaete worms?”, a common occupant of benthic marine sediments worldwide. To achieve this I investigated the biological effects of microplastic ingestion using two species of benthic dwelling worms by assessing microplastic ingestion in natural populations, the effect of plastic shape on toxicity and its subsequent effect on individual fitness.

The data collected demonstrates microplastic ingestion occurs in wild populations of *Hediste diversicolor*, with no apparent influence of local urbanisation on the amount consumed across the three study sites used here. Microfibers were the most commonly found item in worm tissues across these three populations (see chapter 2), an observation that is commonly reported in the gut contents of numerous phyla (Davidson & Dudas, 2016; (Carreras-Colom et al., 2018; Compa et al., 2018; Horton et al., 2018) and in sediment samples worldwide (Woodall et al., 2014; Claessens et al., 2011; Graca et al., 2017; Reed et al., 2018). As the majority of experimental work researching the impacts of microplastic uses exposure to beads, and with little ecotoxicological data available for the more commonly found microfibers, an experimental assessment of the relative biological responses of polychaetes to these different polymer shapes was performed. My data shows clearly that *H. diversicolor* individuals, exposed to microfiber spiked sediments for 48 hrs, have greater oxidative stress responses compared to individuals exposed to beads, which concequently caused celluar damage in the form of lipid peroxidation due to reduced SOD activity.

Taken together, these data highlight the necessity for microplastic toxicity research to proportionally shift focus from assessing the impacts of spherical particles, which are rarely reported in environmental samples, to plastics more

fibrous in form. Such data will better our understanding of the impacts of microplastic pollution in the marine environment. The harmful, but sub-lethal oxidative damage, observed here in chapter 3, could potentially increase an individual's energetic demand for cellular maintenance and repair which consequently may impact individual fitness. We, therefore, went on to assess if the presence of microplastic negativity impacts an individual's reproductive output (see chapter 4). We found *Ophryotrocha labronica* adult mating pairs exposed to 10 - 40 µm PA beads appeared to have higher faecal pellet production, produced less offspring and had significantly smaller eggs compared to unexposed mating pairs which ultimately could lead to deleterious impacts at the population level.

This project provides some of the first evidence for microplastic ingestion in wild populations of *H. diversicolor* and provides direct evidence that ingested fibres may have a greater negative impact compared to that of beads. It also adds to the growing paradigm that microplastic ingestion may have deleterious effects on fitness, as has been demonstrated previously in Cole et al., (2015), Ogonowski et al., (2016) and Sussarellu et al., (2016). Due to the fact microfibers had a greater impact on worms at the cellular level than that of beads in this project, it would be interesting to assess how longer-term exposures to fibres also impact fitness to see if the effect is greater here too. Assessing this, using longer-term exposes to lower levels of fibres would be the most environmentally realistic laboratory-based experiment to do. However, these types of experiment are more logistically challenging.

This data, taken as a whole, shows that at concentrations higher than those found normally in the environment, the biological impacts on the worms were small sub-lethal changes in the form of oxidative stress, altered feeding, and reduced fecundity. Oxidative stress can result from exposure to natural changes in environmental conditions, such as temperature, pH and UV (Lesser, 2006; Tasselli et al., 2017; Glippa et al., 2018) and therefore is likely something organisms are naturally adapted to deal with. These responses might be taken to suggest that microplastic pollution poses only a small risk to marine life. Such evidence, however, does not fit with the current public perception, especially, in terms of the attention plastics has had in the media over the last year compared to that of other environmental issues, which the media can exaggerate. Although

such attention has created much public awareness with positive outcomes, such as with 5p plastic carrier bag charges, banning microbeads in cosmetics and the up-sale of reusable coffee cups, it still could distract the public eye and potential funding from other pressing issues (as discussed in Backhaus & Wagner, (2018)).

Despite the recent increase in research effort in the field, there are still significant gaps in our understanding of the true environmental impacts of microplastic pollution. This is due, however, to a lack of data on long-term exposures to realistic levels of plastic that adequately assess the different factors that potentially influence its effects, such as shape, size, polymer typer and time in the marine environment. Thus the subject is missing important information on how these impacts may build-up over time. However, changes in approach are possible. For example, as seen in the field of ocean acidification where research moved from short-term projects to much longer and more environmentally realistic exposures as the research field progressed with time. This could be how the field of microplastic reseach will move forward into the future.

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